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Purified BMP-9 proteins and processes for producing them are disclosed. The proteins may be used in the treatment of bone and cartilage defects and in wound healing and related tissue repair, and in hepatic growth and function.

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BMP-9 COMPOSITIONS

The present invention relates to a novel family of purified proteins designated BMP-9 proteins and processes for obtaining them. These proteins may be used to induce bone and/or cartilage formation, in wound healing and tissue repair, and in hepatic growth and function.

The murine BMP-9 DNA sequence (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) are set forth in Figure 1. Human BMP-9 sequence is set forth in Figure 3 (SEQ ID NO:8 and SEQ ID NO:9). It is contemplated that BMP-9 proteins are capable of inducing the formation of cartilage and/or bone. BMP-9 proteins may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below.

Murine BMP-9 is characterized by comprising amino acid #319 to #428 of Figure 1 (SEQ ID NO:2 amino acids #1-110). Murine BMP-9 may be produced by culturing a cell transformed with a DNA sequence comprising nucleotide #610 to nucleotide #1893 as shown in Figure 1 (SEQ ID NO:1) and recovering and purifying from the culture medium a protein characterized by the amino acid sequence comprising amino acid #319 to #428 as shown in Figure 1 (SEQ ID NO:2) substantially free from other proteinaceous materials with which it is co-produced.

Human BMP-9 is expected to be homologous to murine BMP-9 and is characterized by comprising amino acid #1 (Ser, Ala, Gly) to #110 of Figure 3 (SEQ ID NO:9) (Arg). The invention includes methods for obtaining the DNA sequences encoding human BMP-9. This method entails utilizing the murine BMP-9 nucleotide sequence or portions thereof to design probes to screen libraries for the human gene or fragments thereof using standard techniques. Human BMP-9 may be produced by culturing a cell transformed with the BMP-9 DNA sequence and recovering and purifying BMP-9 from the culture medium. The expressed protein is isolated, recovered, and purified from the culture medium.

The purified expressed protein is substantially free from other proteinaceous materials with which it is co-produced, as well as from other contaminants. The recovered purified protein is contemplated to exhibit cartilage and/or bone formation activity. The proteins of the invention may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below.

Human BMP-9 may be produced by culturing a cell transformed with a DNA sequence comprising nucleotide #124 to #453 as shown in SEQ ID NO:8 and recovering and purifying from the culture medium a protein characterized by the amino acid sequence of SEQ ID NO:9 from amino acid #1 to amino acid #110 substantially free from other proteinaceous materials with which it is co-produced.

Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle or carrier. BMP-9 compositions of the invention may be used in the formation of cartilage. These compositions may further be utilized for the formation of bone. BMP-9 compositions may also be used for wound healing and tissue repair. Compositions of the invention may further include at least one other therapeutically useful agent such as the BMP proteins BMP-1, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, and BMP-7 disclosed for instance in PCT Publication Nos. WO88/00205, WO89/10409, and WO90/11366, and BMP-8, disclosed in U.S. Application Serial No. 07/641,204 filed January 15, 1991, Serial No. 07/525,357 filed May 16, 1990, and Serial No. 07/800,364 filed November 20, 1991.

The compositions of the invention may comprise, in addition to a BMP-9 protein, other therapeutically useful agents including growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF-α and TGF-β), and insulin-like growth factor (IGF). The compositions may also include an appropriate matrix for instance, for supporting the composition and providing a surface for bone and/or cartilage growth. The matrix may provide slow release of the osteoinductive protein and/or the appropriate environment for presentation thereof.

The BMP-9 compositions may be employed in methods for treating a number of bone and/or cartilage defects, periodontal disease and various types of wounds. These methods, according to the invention, entail administering to a patient needing such bone and/or cartilage formation wound healing or tissue repair, an effective amount of a BMP-9 protein. These methods may also entail the administration of a protein of the invention in conjunction with at least one of the novel BMP proteins disclosed in the co-owned applications described above. In addition, these methods may also include the administration of a BMP-9 protein with other growth factors including EGF, FGF, TGF-α, TGF-β, and IGF.

Still a further aspect of the invention are DNA sequences coding for expression of a BMP-9 protein. Such sequences include the sequence of nucleotides in a 5′ to 3′ direction illustrated in Figure 1 (SEQ ID NO:1) and Figure 3 (SEQ ID NO:8) or DNA sequences which hybridize under stringent conditions with the DNA sequences of Figure 1 or 3 and encode a protein having the ability to induce the formation of cartilage and/or bone. Finally, allelic or other variations of the sequences of Figure 1 or 3, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

A further aspect of the invention includes vectors comprising a DNA sequence as described above in operative association with an expression control sequence therefor. These vectors may be employed in a novel process for producing a BMP-9 protein of the invention in which a cell line transformed with a DNA sequence encoding a BMP-9 protein in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and a BMP-9 protein is recovered and purified therefrom. This process may employ a number of known cells both prokaryotic and eukaryotic as host cells for expression of the polypeptide.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 comprises DNA sequence and derived amino acid sequence of murine BMP-9 from clone ML14a further described below.

FIG. 2 comprises DNA sequence and derived amino acid sequence of human BMP-4 from lambda U2OS-3 ATCC #40342.

FIG. 3 comprises DNA sequence and derived amino acid sequence of human BMP-9 from λ FIX/H6111 ATCC #75252.

FIG. 4 sets forth articular cartilage assay sulfate incorporation results.

FIG. 5 sets forth results of specific BMP-9 binding to HepG2 cells.

FIG. 6 sets forth results of stimulation of HepG2 cell proliferation by BMP-9.

FIG. 7 sets forth the results of stimulation of primary rat hepatocytes by BMP-9.

DETAILED DESCRIPTION OF THE INVENTION

The murine BMP-9 nucleotide sequence (SEQ ID NO:1) and encoded amino acid sequence (SEQ ID NO:2) are depicted in Figure 1. Purified murine BMP-9 proteins of the present invention are produced by culturing a host cell transformed with a DNA sequence comprising the DNA coding sequence of Figure 1 (SEQ ID NO:1) from nucleotide #610 to nucleotide #1893 and recovering and purifying from the culture medium a protein which contains the amino acid sequence or a substantially homologous sequence as represented by amino acid #319 to #428 of Figure 1 (SEQ ID NO:2). The BMP-9 proteins recovered from the culture medium are purified by isolating them from other proteinaceous materials from which they are co-produced and from other contaminants present.

Human BMP-9 nucleotide and amino acid sequence is depicted in SEQ ID No: 8 and 9. Mature human BMP-9 is expected to comprise amino acid #1 (Ser, Ala, Gly) to #110 (Arg).

Human BMP-9 may be produced by culturing a cell transformed with a DNA sequence comprising nucleotide #124 to #453 as shown in SEQ ID NO:8 and recovering and purifying from the culture medium a protein characterized by the amino acid sequence of SEQ ID NO:9 from amino acid #1 to amino acid #110 substantially free

from other proteinaceous materials with which it is co-produced.

BMP-9 proteins may be characterized by the ability to induce the formation of cartilage. BMP-9 proteins may be further characterized by the ability to induce the formation of bone. BMP-9 proteins may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below.

The BMP-9 proteins provided herein also include factors encoded by the sequences similar to those of Figure 1 and 3 (SEQ ID NOS:1 and 8), but into which modifications are naturally provided (e.g., allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Figure 1 of Figure 3 (SEQ ID NOS:2 and 9). These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with bone growth factor polypeptides of Figure 1 and Figure 3 may possess bone growth factor biological properties in common therewith. Thus, they may be employed as biologically active substitutes for naturally-occurring BMP-9 and other BMP-9 polypeptides in therapeutic processes.

Other specific mutations of the sequences of BMP-9 proteins described herein involve modifications of glycosylation sites. These modifications may involve O-linked or N-linked glycosylation sites. For instance, the absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at asparagine-linked glycosylation recognition sites. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified

tripeptide sequence.

The present invention also encompasses the novel DNA sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for BMP-9 proteins. These DNA sequences include those depicted in Figure 1 or Figure 3 (SEQ ID NOS:1 and 8) in a 5′ to 3′ direction and those sequences which hybridize thereto under stringent hybridization conditions [see, T. Maniatis et al., Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] and encode a protein having cartilage and/or bone inducing activity.

Similarly, DNA sequences which code for BMP-9 proteins coded for by the sequences of Figure 1 or Figure 3, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel factors described herein. Variations in the DNA sequences of Figure 1 or Figure 3 (SEQ ID NOS:1 and 8) which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the polypeptides encoded are also encompassed in the invention.

Another aspect of the present invention provides a novel method for producing BMP-9 proteins. The method of the present invention involves culturing a suitable cell line, which has been transformed with a DNA sequence encoding a BMP-9 protein of the invention, under the control of known regulatory sequences. The transformed host cells are cultured and the BMP-9 proteins recovered and purified from the culture medium. The purified proteins are substantially free from other proteins with which they are co-produced as well as from other contaminants.

Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening, product production and purification are known in the art. See e.g., Gething and Sambrook, Nature 293:620-625 (1981), or alternatively, Kaufman et al., Mol. Cell.

Biol. 5(7):1750-1759 (1985) or Howley et al., U.S. Patent No. 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. The mammalian cell CV-1 may also be suitable.

Bacterial cells may also be suitable hosts. For example, the various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See e.g., Miller et al., Genetic Engineering 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in the method of expression of these novel BMP-9 polypeptides. Preferably the vectors contain the full novel DNA sequences described above which encode the novel factors of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the BMP-9 protein sequences. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Regulatory sequences for such vectors are known to those skilled in the art and may be selected depending upon the host cells. Such selection is routine and does not form part of the present invention.

A protein of the present invention, which induces cartilage and/or bone formation in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage defects in humans and other animals. Such a preparation employing a BMP-9 protein may have prophylactic use

in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. A BMP-9 protein may be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. BMP-9 polypeptides of the invention may also be useful in the treatment of osteoporosis. BMP-9 may be used in cartilage defect repair and prevention/ reversal of osteoarthritis. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See e.g., European Patent Application Nos. 148,155 and 169,016 for discussions thereof.

The proteins of the invention may also be used in wound healing and related tissue repair. The types of wounds include, but are not limited to burns, incisions and ulcers. (See e.g., PCT Publication No. WO84/01106 for discussion of wound healing and related tissue repair).

It is further contemplated that proteins of the invention may increase neuronal survival and therefore be useful in transplantation and treatment of conditions exhibiting a decrease in neuronal survival.

BMP-9 proteins of the invention may also be useful in hepatic growth and function including repair and regeneration of liver cells. BMP-9 may therefore be used for instance in treatment of conditions exhibiting degeneration of the liver.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to cartilage and/or bone defects or periodontal diseases. The invention further comprises therapeutic methods and compositions for wound healing and tissue repair. Such compositions comprise a therapeutically effective amount of at least one of the BMP-9 proteins of the invention in admixture with a pharmaceutically acceptable vehicle, carrier or matrix.

It is expected that the proteins of the invention may act in concert with or perhaps synergistically with other related proteins and growth factors. Further therapeutic methods and compositions of the invention therefore comprise a therapeutic amount of at least one BMP-9 protein of the invention with a therapeutic amount of at least one of the other BMP proteins disclosed in co-owned applications described above. Such combinations may comprise separate molecules of the BMP proteins or heteromolecules comprised of different BMP moieties. For example, a method and composition of the invention may comprise a disulfide linked dimer comprising a BMP-9 protein subunit and a subunit from one of the "BMP" proteins described above. A further embodiment may comprise a heterodimer of BMP-9 moieties. Further, BMP-9 proteins may be combined with other agents beneficial to the treatment of the bone and/or cartilage defect, wound, or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factors (TGF-α and TGF-β), and insulin-like growth factor (IGF).

The preparation and formulation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art. The therapeutic compositions are also presently valuable for veterinary applications due to the lack of species specificity in BMP proteins. Particularly domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with BMP-9 of the present invention.

The therapeutic method includes administering the composition topically, systemically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than the BMP-9 proteins which may also optionally be included in the

composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the BMP composition in the methods of the invention. Preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering BMP-9 or other BMP proteins to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. The matrix may provide slow release of BMP-9 and/or the appropriate environment for presentation thereof. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the BMP-9 compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the BMP-9 protein, e.g., amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types

of BMP proteins in the composition. The addition of other known growth factors, such as IGF I (insulin like growth factor I), to the final composition, may also effect the dosage. Progress can be monitored by periodic assessment of bone growth and/or repair, for example, x-rays, histomorphometric determinations and tetracycline labeling.

The following examples illustrate practice of the present invention in recovering and characterizing murine BMP-9 protein and employing it to recover the human and other BMP-9 proteins, obtaining the human proteins and expressing the proteins via recombinant techniques.

EXAMPLE I

MURINE BMP-9

750,000 recombinants of a mouse liver cDNA library made in the vector lambdaZAP (Stratagene/Catalog #935302) are plated and duplicate nitrocellulose replicas made. A fragment of human BMP-4 DNA corresponding to nucleotides 1330-1627 of Figure 2 (SEQ ID NO:3) (the human BMP-4 sequence) is ^{32}P-labeled by the random priming procedure of Feinberg et al., Anal. Biochem. 132:6-13 (1983) and hybridized to both sets of filters in SHB at 60°C for 2 to 3 days. Both sets of filters are washed under reduced stringency conditions (4X SSC, 0.1% SDS at 60°C). Many duplicate hybridizing recombinants of various intensities (approximately 92) are noted. 50 of the strongest hybridizing recombinant bacteriophage are plaque purified and their inserts are transferred to the plasmid Bluescript SK (+/-) according to the in vivo excision protocol described by the manufacturer (Stratagene). DNA sequence analysis of several recombinants indicate that they encode a protein homologous to other BMP proteins and other proteins in the TGF-β family. The DNA sequence and derived amino acid sequence of one recombinant, designated ML14a, is set forth in Figure 1. (SEQ ID NO:1)

The nucleotide sequence of clone ML14a contains an open reading frame of 1284 bp, encoding a BMP-9 protein of 428 amino acids. The encoded 428 amino acid BMP-9 protein is contemplated to be the primary translation product as the coding sequence is

preceded by 609 bp of 5′ untranslated sequence with stop codons in all three reading frames. The 428 amino acid sequence predicts a BMP-9 protein with a molecular weight of 48,000 daltons.

Based on knowledge of other BMP proteins and other proteins within the TGF-β family, it is predicted that the precursor polypeptide would be cleaved at the multibasic sequence ARG-ARG-LYS-ARG in agreement with a proposed consensus proteolytic processing sequence of ARG-X-X-ARG. Cleavage of the BMP-9 precursor polypeptide at this location would generate a 110 amino acid mature peptide beginning with the amino acid SER at position #319. The processing of BMP-9 into the mature form is expected to involve dimerization and removal of the N-terminal region in a manner analogous to the processing of the related protein TGF-β [L. E. Gentry et al., Mol. & Cell. Biol. 8:4162 (1988); R. Derynck et al., Nature 316:701 (1985)].

It is contemplated therefore that the mature active species of murine BMP-9 comprises a homodimer of 2 polypeptide subunits, each subunit comprising amino acids #319-#428 with a predicted molecular weight of approximately 12,000 daltons. Further active species are contemplated comprising amino acids #326 - #428 thereby including the first conserved cysteine residue. As with other members of the BMP and TGF-β family of proteins, the carboxy-terminal region of the BMP-9 protein exhibits greater sequence conservation than the more amino-terminal portion. The percent amino acid identity of the murine BMP-9 protein in the cysteine-rich C-terminal domain (amino acids #326 - #428) to the corresponding region of other human BMP proteins and other proteins within the TGF-β family is as follows: BMP-2, 53%; BMP-3, 43%; BMP-4, 53%; BMP-5, 55%; BMP-6, 55%; BMP-7, 53%; Vg1, 50%; GDF-1, 43%; TGF-β1, 32%; TGF-β2, 34%; TGF-β3, 34%; inhibin β(B), 34%; and inhibin β(A), 42%.

EXAMPLE II

HUMAN BMP-9

Murine and human osteoinductive factor genes are presumed to be significantly homologous, therefore the murine coding

sequence or a portion thereof is used as a probe to screen a human genomic library or as a probe to identify a human cell line or tissue which synthesizes the analogous human cartilage and/or bone protein. A human genomic library (Toole et al., supra) may be screened with such a probe, and presumptive positives isolated and DNA sequence obtained. Evidence that this recombinant encodes a portion of the human BMP-9 relies of the murine/human protein and gene structure homologies.

Once a recombinant bacteriophage containing DNA encoding portion of the human cartilage and/or bone inductive factor molecule is obtained, the human coding sequence can be used as a probe to identify a human cell line or tissue which synthesizes BMP-9. Alternatively, the murine coding sequence can be used as a probe to identify such human cell line or tissue. Briefly described, RNA is extracted from a selected cell or tissue source and either electrophoresed on a formaldehyde agarose gel and transferred to nitrocellulose, or reacted with formaldehyde and spotted on nitrocellulose directly. The nitrocellulose is then hybridized to a probe derived from a coding sequence of the murine or human BMP-9. mRNA is selected by oligo (dT) cellulose chromatography and cDNA is synthesized and cloned in lambda gt10 or lambda ZAP by established techniques (Toole et al., supra).

Additional methods known to those skilled in the art may be used to isolate the human and other species' BMP-9 proteins of the invention.

A. ISOLATION OF HUMAN BMP-9 DNA

One million recombinants of a human genomic library constructed in the vector λFIX (Stratagene catalog # 944201) are plated and duplicate nitrocellulose replicas made. Two oligonucleotides probes designed on the basis of nucleotides #1665-#1704 and #1837-#1876 of the sequence set forth in Figure 1 (SEQ ID NO:1) are synthesized on an automated DNA synthesizer. The sequence of these two oligonucleotides is indicated below:

#1: CTATGAGTGTAAAGGGGGTTGCTTCTTCCCATTGGCTGAT

#2: GTGCCAACCCTCAAGTACCACTATGAGGGGATGAGTGTGG

These two oligonucleotide probes are radioactively labeled with

γ^{32}P-ATP and each is hybridized to one set of the duplicate nitrocellulose replicas in SHB at 65°C and washed with 1X SSC, 0.1% SDS at 65°C. Three recombinants which hybridize to both oligonucleotide probes are noted. All three positively hybridizing recombinants are plaque purified, bacteriophage plate stocks are prepared and bacteriophage DNA is isolated from each. The oligonucleotide hybridizing regions of one of these recombinants, designated HG111, is localized to a 1.2 kb Pst I/Xba I fragment. This fragment is subcloned into a plasmid vector (pGEM-3) and DNA sequence analysis is performed. HG111 was deposited with the American Type Culture Collection ATCC, 12301 Parklawn Drive, Rockville, Maryland USA (hereinafter the "ATCC") on June 16, 1992 under the requirements of the Budapest Treaty and designated as ATCC #75252. This subclone is designated pGEM-111. A portion of the DNA sequence of clone pGEM-111 is set forth in Figure 3 (SEQ ID NO:8/HUMAN BMP-9 SEQUENCE). This sequence encodes the entire mature region of human BMP-9 and a portion of the propeptide. It should be noted that this sequence consists of preliminary data. Particularly, the propeptide region is subject to further analysis and characterization. For example, nucleotides #1 through #3 (TGA) encode a translational stop which may be incorrect due to the preliminary nature of the sequence. It is predicted that additional sequences present in both pGEM-111 (the 1.2 kb PstI/XbaI fragment of HG111 subcloned into pGEM) and HG111 encode additional amino acids of the human BMP-9 propeptide region. Based on knowledge of other BMPs and other proteins within the TGF-β family, it is predicted that the precursor polypeptide would be cleaved at the multibasic sequence ARG-ARG-LYS-ARG (amino acids # -4 through # -1 of SEQ ID NO:9) in agreement with a proposed consensus proteolytic processing sequence ARG-X-X-ARG. Cleavage of the human BMP-9 precursor polypeptide at this location would generate a 110 amino acid mature peptide beginning with the amino acid SER at position #1 of SEQ ID NO:9 (encoded by nucleotides #124 through #126 of SEQ ID NO:8). The processing of human BMP-9 into the mature form is expected to involve dimerization and removal of the N-terminal region in a manner

analogous to the processing of the related protein TGF-β [L.E. Gentry et al., Mol. & Cell. Biol. 8:4162 (1988); R. Derynck et al., Nature 316:701 (1985)].

It is contemplated therefore that the mature active species of human BMP-9 comprises a homodimer of two polypeptide subunits, each subunit comprising amino acids #1 through #110 of SEQ ID NO:9, with a predicted molecular weight of 12,000 daltons. Further active species are contemplated comprising amino acids #8 through #110 thereby including the first conserved cysteine residue. As with other members of the BMP and TGF-β family of proteins, the carboxy-terminal portion of the human BMP-9 sequence exhibits greater sequence conservation than the amino-terminal portion. The percent amino acid identity of the human BMP-9 protein in the cysteine-rich C-terminal domain (amino acids #8 through #110) to the corresponding region of other human BMP proteins and other proteins within the TGF-β family is as follows: BMP-2, 52%; BMP-3, 40%; BMP-4, 52%; BMP-5, 55%; BMP-6, 55%; BMP-7, 53%; murine BMP-9, 97%; Vg1, 50%; GDF-1, 44%; TGF-β1, 32%; TGF-β2, 32%; TGF-β3, 32%; inhibin β (B), 35%; and inhibin β (A), 41%. BMP-9 exhibits 80% homology to chick Dorsalin-1, a BMP-like protein cloned from embryonic chick.

EXAMPLE III

ROSEN MODIFIED SAMPATH-REDDI ASSAY

A modified version of the rat bone formation assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. USA 80:6591-6595 (1983) is used to evaluate bone and/or cartilage activity of the BMP proteins. This modified assay is herein called the Rosen-modified Sampath-Reddi assay. The ethanol precipitation step of the Sampath-Reddi procedure is replaced by dialyzing (if the composition is a solution) or diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then redissolved in 0.1 % TFA, and the resulting solution added to 20mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted

subcutaneously in the abdominal thoracic area of 21 - 49 day old male Long Evans rats. The implants are removed after 7 - 14 days. Half of each implant is used for alkaline phosphatase analysis [See, A. H. Reddi et al., Proc. Natl Acad Sci. 69:1601 (1972)].

The other half of each implant is fixed and processed for histological analysis. 1lm glycolmethacrylate sections are stained with Von Kossa and acid fuschin to score the amount of induced bone and cartilage formation present in each implant. The terms +1 through +5 represent the area of each histological section of an implant occupied by new bone and/or cartilage cells and matrix. A score of +5 indicates that greater than 50% of the implant is new bone and/or cartilage produced as a direct result of protein in the implant. A score of +4, +3, +2 and +1 would indicate that greater than 40%, 30%, 20% and 10% respectively of the implant contains new cartilage and/or bone. In a modified scoring method, three non-adjacent sections are evaluated from each implant and averaged. "+/-" indicates tentative identification of cartilage or bone; "+1" indicates >10% of each section being new cartilage or bone; "+2", >25%; "+3", >50%; "+4", ~75%; "+5", >80%. A "-" indicates that the implant is not recovered.

It is contemplated that the dose response nature of the BMP-9 containing samples of the matrix samples will demonstrate that the amount of bone and/or cartilage formed increases with the amount of BMP-9 in the sample. It is contemplated that the control samples will not result in any bone and/or cartilage formation.

As with other cartilage and/or bone inductive proteins such as the above-mentioned "BMP" proteins, the bone and/or cartilage formed is expected to be physically confined to the space occupied by the matrix. Samples are also analyzed by SDS gel electrophoresis and isoelectric focusing followed by autoradiography. The activity is correlated with the protein bands and pI. To estimate the purity of the protein in a particular fraction an extinction coefficient of 1 OD/mg-cm is used as an estimate for protein and the protein is run on SDS

PAGE followed by silver staining or radioiodination and autoradiography.

EXAMPLE IV

EXPRESSION OF BMP-9

In order to produce murine, human or other mammalian BMP-9 proteins, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. The preferred expression system for biologically active recombinant human BMP-9 is contemplated to be stably transformed mammalian cells.

One skilled in the art can construct mammalian expression vectors by employing the sequence of Figure 1 (SEQ ID NO:1) or Figure 3 (SEQ ID NO:8), or other DNA sequences encoding BMP-9 proteins or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol. 2:161-170 (1982)], pJL3, pJL4 [Gough et al., EMBO J. 4:645-653 (1985)] and pMT2 CXM.

The mammalian expression vector pMT2 CXM is a derivative of p91023 (b) (Wong et al., Science 228:810-815 (1985)) differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 CXM have been described (Kaufman, R.J., Proc. Natl. Acad. Sci. USA 82:689-693 (1985)) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5′ splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3′ splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in E. coli.

Plasmid pMT2 CXM is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC #67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin

resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2 CXM is then constructed using loopout/in mutagenesis [Morinaga et al., Biotechnology 84:636 (1984)]. This removes bases 1075 to 1145 relative to the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. In addition it inserts the following sequence:

5′ PO-CATGGGCAGCTCGAG-3′ (SEQ ID NO:5)

at nucleotide 1145. This sequence contains the recognition site for the restriction endonuclease Xho I. A derivative of pMT2CXM, termed pMT23, contains recognition sites for the restriction endonucleases PstI, Eco RI, SalI and XhoI. Plasmid pMT2 CXM and pMT23 DNA may be prepared by conventional methods.

pEMC2b1 derived from pMT21 may also be suitable in practice of the invention. pMT21 is derived from pMT2 which is derived from pMT2-VWF. As described above EcoRI digestion excises the cDNA insert present in pMT-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods.

pMT21 is derived from pMT2 through the following two modifications. First, 76 bp of the 5′ untranslated region of the DHFR cDNA including a stretch of 19 G residues from G/C tailing for cDNA cloning is deleted. In this process, a XhoI site is inserted to obtain the following sequence immediately upstream from DHFR: 5′ -CTGCAGGCGAGCCTGAATTCCTCGAGCCATCATG-3′

PstI Eco RI XhoI

(SEQ ID NO:6)

Second, a unique ClaI site is introduced by digestion with EcoRV and XbaI, treatment with Klenow fragment of DNA polymerase I, and ligation to a ClaI linker (CATCGATG). This deletes a 250 bp segment from the adenovirus associated RNA (VAI) region but does not interfere with VAI RNA gene expression or function. pMT21 is digested with EcoRI and XhoI, and used to derive the vector pEMC2B1.

A portion of the EMCV leader is obtained from pMT2-ECAT1 [S.K. Jung et al., J. Virol 63:1651-1660 (1989)] by digestion with Eco RI and PstI, resulting in a 2752 bp fragment. This fragment is digested with TaqI yielding an Eco RI-TaqI fragment

of 508 bp which is purified by electrophoresis on low melting agarose gel. A 68 bp adapter and its complementary strand are synthesized with a 5′ TaqI protruding end and a 3′ XhoI protruding end which has the following sequence:

5′-CGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTT
TaqI

GAAAAACACGATTGC-3′
XhoI (SEQ ID NO:7)

This sequence matches the EMC virus leader sequence from nucleotide 763 to 827. It also changes the ATG at position 10 within the EMC virus leader to an ATT and is followed by a XhoI site. A three way ligation of the pMT21 Eco RI-XhoI fragment, the EMC virus EcoRI-TaqI fragment, and the 68 bp oligonucleotide adapter TaqI-XhoI adapter resulting in the vector pEMC2β1.

This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the majority of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the adenovirus VA I gene, DHFR and β-lactamase markers and an EMC sequence, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells.

The construction of vectors may involve modification of the BMP-9 DNA sequences. For instance, BMP-9 cDNA can be modified by removing the non-coding nucleotides on the 5′ and 3′ ends of the coding region. The deleted non-coding nucleotides may or may not be replaced by other sequences known to be beneficial for expression. These vectors are transformed into appropriate host cells for expression of BMP-9 proteins. One skilled in the art can manipulate the sequences of Figure 1 or Figure 3 (SEQ ID NOS:1 and 8) by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g., ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). The modified BMP-9 coding sequence could then be inserted into a

known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and a BMP-9 protein expressed thereby. For a strategy for producing extracellular expression of BMP-9 proteins in bacterial cells, see e.g., European Patent Application No. EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See e.g., procedures described in published European Patent Application No. 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See e.g., procedures described in published PCT Publication No. WO86/00639 and European Patent Application No. EPA 123,289].

A method for producing high levels of a BMP-9 protein of the invention in mammalian cells may involve the construction of cells containing multiple copies of the heterologous BMP-9 gene. The heterologous gene is linked to an amplifiable marker, e.g., the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol. 159:601-629 (1982). This approach can be employed with a number of different cell types.

For example, a plasmid containing a DNA sequence for a BMP-9 of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol. 2:1304 (1982)] can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by various methods including calcium phosphate coprecipitation and transfection, electroporation or protoplast fusion. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (e.g., sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as

described in Kaufman et al., Mol Cell Biol. 5:1750 (1983). Transformants are cloned, and biologically active BMP-9 expression is monitored by the Rosen-modified Sampath - Reddi rat bone formation assay described above in Example III. BMP-9 expression should increase with increasing levels of MTX resistance. BMP-9 polypeptides are characterized using standard techniques known in the art such as pulse labeling with [35S] methionine or cysteine and polyacrylamide gel electrophoresis. Similar procedures can be followed to produce other related BMP-9 proteins.

A. BMP-9 VECTOR CONSTRUCTION

In order to produce human BMP-9 proteins of the invention DNA sequences encoding the mature region of the human BMP-9 protein may be joined to DNA sequences encoding the propeptide region of the murine BMP-9 protein. This murine/human hybrid DNA sequence is inserted into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. The construction of this murine/human BMP-9 containing expression plasmid is described below.

A derivative of the human BMP-9 sequence (SEQ ID NO:8) comprising the nucleotide sequence from nucleotide #105 to #470 is specifically amplified. The following oligonucleotides are utilized as primers to allow the amplification of nucleotides #105 to #470 of the human BMP-9 sequence (SEQ ID NO:8) from clone pGEM-111 described above.

#3 ATCGGGCCCCTTTTAGCCAGGCGGAAAAGGAG

#4 AGCGAATTCCCCGCAGGCAGATACTACCTG

This procedure generates the insertion of the nucleotide sequence ATCGGGCCCCT immediately preceding nucleotide #105 and the insertion of the nucleotide sequence GAATTCGCT immediately following nucleotide #470. The addition of these sequences results in the creation of an Apa I and EcoR I restriction endonuclease site at the respective ends of the specifically amplified DNA fragment. The resulting 374 bp Apa I/EcoR I fragment is subcloned into the plasmid vector pGEM-7Zf(+)

(Promega catalog# p2251) which has been digested with Apa I and EcoR I. The resulting clone is designated phBMP9mex-1.

The following oligonucleotides are designed on the basis of murine BMP-9 sequences (SEQ ID NO:1) and are modified to facilitate the construction of the murine/human expression plasmid referred to above:

#5

GATTCCGTCGACCACCATGTCCCCTGGGGCCTGGTCTAGATGGATACACAGCTGTGGGGCC

#6 CCACAGCTGTGTATCCATCTAGACCAGGCCCCAGGGGACATGGTGGTCGACG

These oligonucleotides contain complimentary sequences which upon addition to each other facilitate the annealing (base pairing) of the two individual sequences, resulting in the formation of a double stranded synthetic DNA linker (designated LINK-1) in a manner indicated below:

```
      1   5   10        20        30        40        50        60
      |   |   |         |         |         |         |         |
#5GATTCCGTCGACCACCATGTCCCCTGGGGCCTGGTCTAGATGGATACACAGCTGTGGGGCC
        GCAGCTGGTGGTACAGGGGACCCCGGACCAGATCTACCTATGTGTCGACACC #6
```

This DNA linker (LINK-1) contains recognition sequences of restriction endonucleases needed to facilitate subsequent manipulations required to construct the murine/human expression plasmid, as well as sequences required for maximal expression of heterologous sequences in mammalian cell expression systems. More specifically (referring to the sequence numbering of oligonucleotide #5/LINK-1): nucleotides #1-#11 comprise recognition sequences for the restriction endonucleases BamH I and Sal I, nucleotides #11-#15 allow for maximal expression of heterologous sequences in mammalian cell expression systems, nucleotides #16-#31 correspond to nucleotides #610-#625 of the murine BMP-9 sequence (SEQ ID NO:1), nucleotides #32-#33 are inserted to facilitate efficient restriction digestion of two adjacent restriction endonuclease sites (Eco0109 I and Xba I), nucleotides #34-#60 correspond to nucleotides #1515-#1541 of the murine BMP-9 sequence (SEQ ID NO:1) except that nucleotide #58 of synthetic oligonucleotide #5 is a G rather than the A which appears at position #1539 of SEQ ID NO:1 (This nucleotide conversion results in the creation of an Apa I restriction endonuclease recognition sequence, without altering the amino

acid sequence it is intended to encode, to facilitate further manipulations of the murine/human hybrid expression plasmid. LINK-1 (the double stranded product of the annealing of oligonucleotides #5 and #6) is subcloned into the plasmid vector pGEM-7Zf(+) which has been digested with the restriction endonucleases Apa I and BamH I. This results in a plasmid in which the sequences normally present between the Apa I and BamH I sites of the pGEM-7Zf(+) plasmid polylinker are replaced with the sequences of LINK-1 described above. The resulting plasmid clone is designated pBMP-9link.

pBMP-9link is digested with the restriction endonucleases BamH I and Xba I resulting in the removal nucleotides #1-#34 of LINK-1 (refer to the numbering of oligo #5). Clone ML14a, which contains an insert comprising the sequence set forth in SEQ ID NO:1, is also digested with the restriction endonucleases BamH I and Xba I resulting in the removal of sequences comprising nucleotides #1-#1515 of SEQ ID NO:1 (murine BMP-9). This BamH I/Xba I fragment of mouse BMP-9 is isolated from the remainder of the ML14a plasmid clone and subcloned into the BamH I/Xba I sites generated by the removal of the synthetic linker sequences described above. The resulting clone is designated p302.

The p302 clone is digested with the restriction endonuclease Eco0109 I resulting in the excision of nucleotides corresponding to nucleotides #621-#1515 of the murine BMP-9 sequence (SEQ ID NO:1) and nucleotides #35-#59 of LINK-1 (refer to numbering of oligonucleotide #5). It should be noted that the Apa I restriction site created in LINK-1 by the A to G conversion described above is a subset of the recognition sequence of Eco0109 I, therefore digestion of p302 with Eco0109 I cleaves at the Apa I site as well as the naturally occurring murine Eco0109 I (location #619-#625 of SEQ ID NO:1) resulting in the excision of a 920 bp Eco0109 I/Eco0109 I (Apa I) fragment comprising the sequences described above. This 920 Eco0109 I/Eco0109 I (Apa I) fragment is isolated from the remainder of the p302 plasmid clone and subcloned into clone pBMP-9link which has been similarly digested with Eco0109 I. It should be noted that the nucleotides GG (#32-#33 of oligonucleotide #5) originally designed to

facilitate a more complete digestion of the two adjacent restriction sites EcoO109 I and Xba I of LINK-1, which is now a part of pBMP-9link (described above), results in the creation of Dcm methylation recognition sequence. The restriction nuclease EcoO109 I is sensitive to Dcm methylation and therefore cleavage of this sequence (nucleotides #25-#31 of oligonucleotide #5/LINK-1) by the restriction endonuclease EcoO109 I is prevented at this site. Therefore the plasmid clone pBMP-9link is cleaved at the Apa I site but not at the EcoO109 I site upon digestion with the restriction endonuclease EcoO109 I as described above, preventing the intended removal of the sequences between the EcoO109 I and Xba I site of LINK-1 (#32-#55 defined by the numbering of oligonucleotide #5). This results in the insertion of the 920 bp EcoO109 I/Apa I fragment at the EcoO109 I (Apa I) site of pBMP-9link. The resulting clone is designated p318.

Clone p318 is digested with the restriction endonucleases Sal I and Apa I, resulting in the excision of sequences comprising nucleotides #6-#56 of LINK-1 (refer to oligo #5 for location), nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1), and nucleotides #35-#60 of LINK-1 (refer to oligo #5 for location). The resulting 972 bp Sal I/Apa I fragment described above is isolated from the remainder of the p318 plasmid clone and will be utilized in subsequent manipulations.

The clone phBMP9mex-1 (described above), which contains DNA sequences which encode the entire mature region and portions of the propeptide of the human BMP-9 protein, is digested with the restriction endonucleases Apa I and EcoR I. This results in the excision of a 374 bp fragment comprising nucleotides #105-#470 of the human BMP-9 sequence (SEQ ID NO:8) and the additional nucleotides of oligonucleotide primers #3 and #4 which contain the recognition sequences for the restriction endonucleases Apa I and EcoR I. This 374 bp Apa I/EcoR I fragment is combined with the 972 bp Sal I/Apa I fragment from p138 (isolation described above) and ligated to the mammalian cell expression plasmid pED6 (a derivative of pEMC2β1) which has been digested with Sal I and EcoR I. The resulting clone is designated p324.

The clone ML14a (murine BMP-9) is digested with EcoO109 I

and Xba I to generate a fragment comprising nucleotides #621-#1515 of SEQ ID NO:1.

The following oligonucleotides are synthesized on an automated DNA synthesizer and combined such that their complimentary sequences can base pair (anneal) with each other to generate a double stranded synthetic DNA linker designated LINK-2:

#7 TCGACCACCATGTCCCCTGG

#8 GCCCCAGGGGACATGGTGG

This double stranded synthetic DNA linker (LINK-2) anneals in such a way that it generates single stranded ends which are compatible to DNA fragments digested with Sal I (one end) or Eco0109 I (the other end) as indicated below:

#7 TCGACCACCATGTCCCCTGG
GGTGGTACAGGGGACCCCG #8

This LINK-2 synthetic DNA linker is ligated to the 895 bp Eco0109 I/Xba I fragment comprising nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1) described above. This results in a 915 bp Sal I/Xba I fragment.

The clone p324 is digested with Sal I/Xba I to remove sequences comprising nucleotides #6-#56 of LINK-1 (refer to oligo #5 for location) and nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1). The sequences comprising nucleotides #35-#60 of LINK-1 (refer to oligo #5 for location) and the sequences comprising the 374 bp Apa I/EcoR I fragment (human BMP-9 sequences) derived from phBMP9mex-1 remain attached to the pED6 backbone. The 915 bp Sal I/Xba I fragment comprising LINK-2 sequences and nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1) is ligated into the p324 clone from which the Sal I to Xba I sequences described above have been removed.

The resulting plasmid is designated BMP-9 fusion and comprises LINK-2, nucleotides #621-#1515 of murine BMP-9 (SEQ ID NO:1), nucleotides #35-#59 of LINK-1 (refer to the numbering of oligonucleotide #5), and the 374 bp Apa I/EcoR I fragment (human BMP-9) derived from clone pBMP9mex-1 (described above) inserted between the Sal I and EcoR I sites of the mammalian cell expression vector pED6.

B. EXPRESSION

BMP-9 fusion is transfected into CHO cells using standard techniques known to those having ordinary skill in the art to create stable cell lines capable of expressing human BMP-9 protein. The cell lines are cultured under suitable culture conditions and the BMP-9 protein is isolated and purified from the culture medium.

In one embodiment, cells are grown in R1 medium based on a 50:50 mix of F12 and DME plus extra non-essential amino acids plus extra biotin and B12 and 10% fetal bovine serum (FBS) and 0.2 çM methotrexate (MTX). Cells are grown up and expanded into roller bottles in this medium using confluent roller bottles. The serum containing growth medium is discarded, the rollers are rinsed with PBS-CMF, and a serum free production medium is added containing additional amino acids plus insulin (5 çg/ml), putrescine (12.9 çM), hydrocortisone (0.2 çM), selenium (29 nM), and PVA (0.6 g/L). Dextran sulfate is used in this CM (at 100 çg/ml). Conditioned medium (CM) is collected at 24 hours and the rollers are refed with fresh serum free medium. Four sequential 24 hour harvest can be collected. Conditioned medium is clarified (floating cells in the CM are removed) for purification by passing the CM through a 5 ç (pass Profile) pore size filter and a 0.22 ç (millipore Duropore) pore size filter.

EXAMPLE V

BIOLOGICAL ACTIVITY OF EXPRESSED BMP-9

To measure the biological activity of the expressed BMP-9 proteins obtained in Example IV above, the proteins are recovered from the cell culture and purified by isolating the BMP-9 proteins from other proteinaceous materials with which they are co-produced as well as from other contaminants. The purified protein may be assayed in accordance with the rat bone formation assay described in Example III.

Purification is carried out using standard techniques known to those skilled in the art. It is contemplated, as with other BMP proteins, that purification may include the use of Heparin sepharose.

In one embodiment, 40 liters of the conditioned media from Example IV-B is titrated to pH 6.9 with concentrated sodium phosphate pH 6.0, and loaded onto Cellufine Sulfate, previously equilibrated with 50 mM sodium phosphate, pH 6.9. The resin is washed with 50 mM sodium phosphate, 0.5 M NaCl, followed by 50 mM sodium phosphate, 0.5 M NaCl, 0.5 M Arg, pH 6.9. BMP-9 is found in the wash as well as the elution, with a lesser amount of contaminants in the elution pool. Cellufine sulfate pools are concentrated and directly loaded onto RP-HPLC for final purification. Each concentrated pool is titrated to pH 3.8 with dilute TFA and loaded onto a 0.46 X 25 cm C_4 reverse phase column running a linear gradient from 30% A (0.1% TFA/H_2O) to 55% B (0.1% TFA/90% Acetonitrile) over 100 minutes. BMP-9 monomer is separated by baseline resolution from BMP-9 dimer. The identity of monomer and dimer pools are confirmed by N-terminal sequencing. Although heterogeneity in the N-terminus is expected sequencing reveals a predominant species Ser-Ala-Gly-Ala beginning with amino acid #1 of SEQ ID NO:9.

Protein analysis is conducted using standard techniques such as SDS-PAGE acrylamide [U.K. Laemmli, Nature 227:680 (1970)] stained with silver [R.R. Oakley et al., Anal. Biochem. 105:361 (1980)] and by immunoblot [H. Towbin et al., Proc. Natl. Acad. Sci. USA 76:4350 (1979)]. BMP-9 is efficiently expressed in CHO cells as a 14kDa nonglycosylated protein when analyzed under reducing conditions. BMP-9 is efficiently secreted within 4 hours of its synthesis.

EXAMPLE VI

A. W-20 BIOASSAY

Use of the W-20 bone marrow stromal cells as an indicator cell line is based upon the conversion of these cells to osteoblast-like cells after treatment with BMP-2 [R. S. Thies et al., "Bone Morphogenetic Protein alters W-20 stromal cell differentiation in vitro", Journal of Bone and Mineral Research 5(2):305 (1990); and R. S. Thies et al., "Recombinant Human Bone Morphogenetic Protein 2 Induces Osteoblastic Differentiation in W-20-17 Stromal Cells", Endocrinology, in press (1992)].

Specifically, W-20 cells are a clonal bone marrow stromal cell line derived from adult mice by researchers in the laboratory of Dr. D. Nathan, Children's Hospital, Boston, MA. BMP-2 treatment of W-20 cells results in (1) increased alkaline phosphatase production, (2) induction of PTH stimulated cAMP, and (3) induction of osteocalcin synthesis by the cells. While (1) and (2) represent characteristics associated with the osteoblast phenotype, the ability to synthesize osteocalcin is a phenotypic property only displayed by mature osteoblasts. Furthermore, to date we have observed conversion of W-20 stromal cells to osteoblast-like cells only upon treatment with BMPs. In this manner, the in vitro activities displayed by BMP treated W-20 cells correlate with the in vivo bone forming activity known for BMPs.

Below two in vitro assays useful in comparison of BMP activities of novel osteoinductive molecules are described.

B. W-20 ALKALINE PHOSPHATASE ASSAY PROTOCOL

W-20 cells are plated into 96 well tissue culture plates at a density of 10,000 cells per well in 200 µl of media (DME with 10% heat inactivated fetal calf serum, 2 mM glutamine and 100 U/ml + 100 µg/ml streptomycin. The cells are allowed to attach overnight in a 95% air, 5% CO_2 incubator at 37°C.

The 200 µl of media is removed from each well with a multichannel pipettor and replaced with an equal volume of test sample delivered in DME with 10% heat inactivated fetal calf serum, 2 mM glutamine and 1% penicillin-streptomycin. Test substances are assayed in triplicate.

The test samples and standards are allowed a 24 hour incubation period with the W-20 indicator cells. After the 24 hours, plates are removed from the 37°C incubator and the test media are removed from the cells.

The W-20 cell layers are washed 3 times with 200 µl per well of calcium/magnesium free phosphate buffered saline and these washes are discarded.

50 µl of glass distilled water is added to each well and the assay plates are then placed on a dry ice/ethanol bath for quick

freezing. Once frozen, the assay plates are removed from the dry ice/ethanol bath and thawed at 37°C. This step is repeated 2 more times for a total of 3 freeze-thaw procedures. Once complete, the membrane bound alkaline phosphatase is available for measurement.

50 µl of assay mix (50 mM glycine, 0.05% Triton X-100, 4 mM $MgCl_2$, 5 mM p-nitrophenol phosphate, pH = 10.3) is added to each assay well and the assay plates are then incubated for 30 minutes at 37°C in a shaking waterbath at 60 oscillations per minute.

At the end of the 30 minute incubation, the reaction is stopped by adding 100 µl of 0.2 N NaOH to each well and placing the assay plates on ice.

The spectrophotometric absorbance for each well is read at a wavelength of 405 nanometers. These values are then compared to known standards to give an estimate of the alkaline phosphatase activity in each sample. For example, using known amounts of p-nitrophenol phosphate, absorbance values are generated. This is shown in Table I.

Table I
Absorbance Values for Known Standards of P-Nitrophenol Phosphate

P-nitrophenol phosphate umoles	Mean absorbance (405 nm)
0.000	0
0.006	0.261 +/- .024
0.012	0.521 +/- .031
0.018	0.797 +/- .063
0.024	1.074 +/- .061
0.030	1.305 +/- .083

Absorbance values for known amounts of BMP-2 can be determined and converted to µmoles of p-nitrophenol phosphate cleaved per unit time as shown in Table II.

Table II
Alkaline Phosphatase Values for W-20 Cells
Treating with BMP-2

BMP-2 concentration ng/ml	Absorbance Reading 405 nmeters	umoles substrate per hour
0	0.645	0.024
1.56	0.696	0.026
3.12	0.765	0.029
6.25	0.923	0.036
12.50	1.121	0.044
25.0	1.457	0.058
50.0	1.662	0.067
100.0	1.977	0.080

These values are then used to compare the activities of known amounts of BMP-9 to BMP-2.

C. OSTEOCALCIN RIA PROTOCOL

W-20 cells are plated at 10^6 cells per well in 24 well multiwell tissue culture dishes in 2 mls of DME containing 10% heat inactivated fetal calf serum, 2 mM glutamine. The cells are allowed to attach overnight in an atmosphere of 95% air 5% CO_2 at 37°C.

The next day the medium is changed to DME containing 10% fetal calf serum, 2 mM glutamine and the test substance in a total volume of 2 ml. Each test substance is administered to triplicate wells. The test substances are incubated with the W-20 cells for a total of 96 hours with replacement at 48 hours by the same test medias.

At the end of 96 hours, 50 µl of the test media is removed from each well and assayed for osteocalcin production using a radioimmunoassay for mouse osteocalcin. The details of the assay are described in the kit manufactured by Biomedical Technologies Inc., 378 Page Street, Stoughton, MA 02072. Reagents for the assay are found as product numbers BT-431 (mouse osteocalcin standard), BT-432 (Goat anti-mouse Osteocalcin), BT-431R (iodinated mouse osteocalcin), BT-415 (normal goat serum) and BT-414 (donkey anti goat IgG). The RIA for osteocalcin synthesized by W-20 cells in response to BMP treatment is carried out as

described in the protocol provided by the manufacturer.

The values obtained for the test samples are compared to values for known standards of mouse osteocalcin and to the amount of osteocalcin produced by W-20 cells in response to challenge with known amounts of BMP-2.

Table III
Osteocalcin Synthesis by W-20 Cells

BMP-2 Concentration ng/ml	Osteocalcin Synthesis ng/well
0	0.8
2	0.9
4	0.8
8	2.2
16	2.7
31	3.2
62	5.1
125	6.5
250	8.2
500	9.4
1000	10.0

EXAMPLE VII

ARTICULAR CARTILAGE ASSAY

The effect of BMP-9 on articular cartilage proteoglycan and DNA synthesis is assayed to determine if BMP-9 is involved in the regulation of metabolism of differentiated articular cartilage.

Articular cartilage explants from calf carpal joints are maintained in DMEM with 50 μg/ml ascorbate, 4 mM glutamine and antibiotics for 3 days. Cytokines (rhBMP-2, rhBMP-4, rhBMP-6 and rhBMP-9, IGF-1, bFGF (1-1000 ng/ml), and TGFβ (1-100 ng/ml)) are added to the medium and culture is continued for 3 more days. Medium is changed daily. Twenty-four hours prior to harvest, explants are pulsed with 50 μCi/ml $^{35}SO_4$ or 25 μCi/ml ^{3}H-thymidine. Explants are solubilized and separation of free isotope is performed by gel chromatography. Total DNA of each explant is measured by a spectrophotometric assay. BMP-9 stimulates proteoglycan synthesis above control levels at a dose of 10 ng/ml ($p<0.05$).

BMP-4, BMP-6, BMP-9 and TGFβ are significantly more active in stimulating proteoglycan synthesis at 100 ng/ml. At the

highest doses of cytokine tested (1 μg/ml), proteoglycan synthesis by explants exposed to all cytokines are significantly greater ($p<0.05$) than that by control explants. Sulfate incorporation results are set forth in Figure 4.

Recombinant human BMP-9 stimulates alkaline phosphatase activity in the osteoprogenitor cell line, W-20-17, in a dose responsive manner with an ED_{50} of 4 ng/ml. In vivo, high doses are rhBMP-9 induce ectopic bone formation, with 25 μg/implant of rhBMP-9 inducing cartilage and bone tissue after 10 days of implantation.

EXAMPLE VIII

STIMULATION OF LIVER CELLS

It is contemplated that BMP-9 may be used in liver repair or regeneration. Through the use of whole embryo sections or whole mount techniques, expression of mRNA in multiple tissue is screened simultaneously. In the 11.5 dpc mouse embryo, BMP-9 mRNA localizes exclusively to the developing liver. It is contemplated that BMP-9, like all other BMPs studied to date, acts as a local regulator of cell growth and differentiation, therefore this very specific expression pattern suggests liver as a BMP-9 target tissue.

BMP-9 responsiveness in parenchymal liver cells is tested by screening four liver cell lines for their ability to bind iodinated, CHO-derived BMP-9. The four liver cell lines, HepG2 (ATCC HB8065), NMuli (ATCC CRL1638), Chang and NCTC1469 (ATCC CCL9.1), all specifically bind ^{125}I-BMP-9 to some extent, with HepG2 and NCTC1469 cell lines exhibiting the highest degree of binding. Specific binding of BMP-9 to HepG2 cells is carried out by incubating HepG2 cells grown to confluence in Dulbecco's Modified Eagle's Medium (DME) containing 10% heat-inactivated fetal calf serum (FCS) on gelatinized 6 well plates with 2 ng/ml ^{125}I-BMP-9 and increasing concentrations of unlabelled BMP-9 in binding buffer (136.9 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.64 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 25 mM HEPES and 0.5% BSA, pH 7.4) for 20 hours at 4°C to achieve binding equilibrium. This incubation follows a one hour

preincubation at 37°C in binding buffer alone. For crosslinking experiments, the cells were incubated with 500 μM disuccinimidyl suberate for 20 minutes at 4°C following binding. Cell extracts were analyzed on SDS-PAGE. As shown in Figure 5, HepG2 cells expressed abundant high affinity receptors for BMP-9. Scatchard analysis of these binding data resulted in a curvilinear plot, with approximately 10,000 high affinity receptors per cell. These receptors exhibited a K_d of 0.3 nM. The curvilinear nature of the Scatchard plot indicates negative cooperativity among BMP-9 receptors or that HepG2 cells express at least two populations of BMP-9 receptors with different affinities. Crosslinking analysis on HepG2 cells with ^{125}I-BMP-9 yields two binding proteins of apparent molecular weights of 54 and 80 kD. Crosslinked ligand/receptor complexes were observed at 78 and 100 kD under nonreducing conditions, and 67 and 94 kD under reducing conditions. Subtracting the molecular weight of the BMP-9 dimer and monomer, respectively, it is estimated that these BMP-9 receptor proteins have molecular weights of approximately 54 and 80 kD. The K_d of the high affinity binding sites for BMP-9 is estimated to be approximately 270 pM for HepG2 cells. To test the binding specificity of the receptors for BMP-9, HepG2 cells were incubated with ^{125}I-BMP-9 and a 250-fold excess of different unlabeled ligands. The BMP-9 receptors expressed on HepG2 cells show only limited crossreactivity with BMPs 2 and 4, and no crossreactivity with BMPs 3, 5, 6, 7, 12 and 2/6, or with TGF-ß1 or TGF-ß2.

As a first indication of BMP-9 effects on confluent, serum starved HepG2 cells, cell proliferation is examined as determined by ^{3}H-thymidine incorporation and cell counting. HepG2 cells are plated at 10^6 cells/well in 96 well plates and cultured for 48 hours in DME/0.1% FCS to synchronize the cell cycle are treated for 24 hours with or without BMP-9 in the presence of 0.1% FCS. In ^{3}H-thymidine incorporation assays, ^{3}H-thymidine was included during the last 4 hours of treatment and cellular DNA was collected with a 96 well plate cell harvester. Proliferation was assayed by quantifying ethanol-precipitable ^{3}H-thymidine incorporation by liquid scintillation counting. For cell

counting assays, cells were trypsinized and counted with a hemacytometer. Primary rat hepatocytes isolated from male Fischer 344 rats (Charles River, Wilmington, MA) by collagenase digestion as perviously described [Michalopoulos et al., Cancer Res. 42:4673-4682 (1982)] are plated on collagen-coated plates at subconfluence (5,000-10,000 cells/cm^2) in serum-free media as described in Michalopoulos et al., Cancer Res. 42:4673-4682 (1982) and treated with or without rhBMP-9 for 36 hours. ^{3}H-thymidine was included throughout the treatment period and incorporated ^{3}H-thymidine was quantified as described by Anscher et al., New England J. Med. 328:1592-1598 (1993). BMP-9 stimulates ^{3}H-thymidine incorporation in HepG2 cells approximately five fold. This effect is confirmed by a stimulatory effect of BMP-9 in cell counting experiments. As shown in Figure 6, BMP-9 stimulated ^{3}H-thymidine incorporation in HepG2 cells in a dose-responsive manner. The ED_{50} for this effect was estimated at 10 ng/ml BMP-9. This ED_{50} value is consistent with the estimated binding affinity (K_d = 0.3 nM = 8 ng/ml), suggesting that this biological effect is mediated by the described BMP-9 receptors.

To determine if this proliferative effect of BMP-9 was unique to the HepG2 liver tumor cell line, primary rat hepatocytes were tested for effects of BMP-9 on ^{3}H-thymidine incorporated as shown in Figure 7. BMP-9 stimulated ^{3}h-thymidine incorporation in primary hepatocytes, although not as markedly as EGF. This stimulatory effect is cell density-dependent in primary rat hepatocytes. While subconfluent cells exhibited a stimulation in response to BMP-9, confluent primary hepatocytes did not. As indicatedin Figure 7, in contrast to rhBMP-9, TGF-β1 was inhibitory, not stimulatory on primary rat hepatocytes.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rosen, Vicki A.
Wozney, John M.
Celeste, Anthony J.

(ii) TITLE OF INVENTION: BMP-9 COMPOSITIONS

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genetics Institute, Inc.
(B) STREET: Legal Affairs - 87 CambridgePark Drive
(C) CITY: Cambridge
(D) STATE: MA
(E) COUNTRY: US
(F) ZIP: 02140

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kapinos, Ellen J.
(B) REGISTRATION NUMBER: 32,245
(C) REFERENCE/DOCKET NUMBER: GI 5186C-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 876-1210
(B) TELEFAX: (617) 876-5851

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2447 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus
(B) STRAIN: C57B46xCBA
(F) TISSUE TYPE: liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Mouse liver cDNA
(B) CLONE: ML14A

(viii) POSITION IN GENOME:
(C) UNITS: bp

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1564..1893

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 610..1896

(ix) FEATURE:
(A) NAME/KEY: mRNA
(B) LOCATION: 1..2447

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCTAGGAGAT TTGTTGATCC AATAAATATG ATTAGGGAAA CAATTATTAG GGTTCATGTT     240

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 -240                -235                -230

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-225                -220                -215                -210

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 -160                 -155                 -150

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-145                 -140                 -135                 -130

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TGTCCTCAGG GAGAACAGCA TTGCTGTTCC TGTGCCTCAA GCTCCCAGCT GACTCTCCTG    2323

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 428 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1954 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Osteosarcoma Cell Line
(H) CELL LINE: U-2OS

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: U2OS cDNA in Lambda gt10
(B) CLONE: Lambda U2OS-3

(viii) POSITION IN GENOME:
(C) UNITS: bp

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 403..1629

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1279..1626

(ix) FEATURE:
(A) NAME/KEY: mRNA
(B) LOCATION: 9..1934

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 65                  70                  75                  80

TGT GTG CCC ACT GAA CTG AGT GCC ATC TCC ATG CTG TAC CTG GAT GAG    1566
Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu
                 85                  90                  95

TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG ATG GTA GTA GAG GGA    1614
Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met Val Val Glu Gly
            100                 105                 110

TGT GGG TGC CGC TGAGATCAGG CAGTCCTTGA GGATAGACAG ATATACACAC        1666
Cys Gly Cys Arg
        115

CACACACACA CACCACATAC ACCACACACA CACGTTCCCA TCCACTCACC CACACACTAC  1726

ACAGACTGCT TCCTTATAGC TGGACTTTTA TTTAAAAAAA AAAAAAAAAA AATGGAAAAA  1786

ATCCCTAAAC ATTCACCTTG ACCTTATTTA TGACTTTACG TGCAAATGTT TTGACCATAT  1846

TGATCATATA TTTTGACAAA ATATATTTAT AACTACGTAT TAAAAGAAAA AAATAAAATG  1906

AGTCATTATT TTAAAAAAAA AAAAAAAACT CTAGAGTCGA CGGAATTC               1954
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 408 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ile Pro Gly Asn Arg Met Leu Met Val Val Leu Leu Cys Gln Val
-292     -290                -285                -280

Leu Leu Gly Gly Ala Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys
    -275                -270                -265

Lys Lys Val Ala Glu Ile Gln Gly His Ala Gly Gly Arg Arg Ser Gly
-260                 -255                -250                -245

Gln Ser His Glu Leu Leu Arg Asp Phe Glu Ala Thr Leu Leu Gln Met
                -240                -235                -230

Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys Ser Ala Val Ile Pro
            -225                -220                -215

Asp Tyr Met Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu Glu Glu Glu
        -210                -205                -200

Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala Ser
    -195                -190                -185

Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn
-180                 -175                -170                -165

Ile Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu
                -160                -155                -150

Ser Ser Ile Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg Leu
            -145                -140                -135
```

```
Phe Arg Glu Gln Val Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His
      -130                 -125                 -120

Arg Ile Asn Ile Tyr Glu Val Met Lys Pro Pro Ala Glu Val Val Pro
   -115                 -110                 -105

Gly His Leu Ile Thr Arg Leu Leu Asp Thr Arg Leu Val His His Asn
-100                 -95                  -90                  -85

Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro Ala Val Leu Arg Trp
                -80                  -75                  -70

Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu Val Thr His
          -65                  -60                  -55

Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser Arg
      -50                  -45                  -40

Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu
   -35                  -30                  -25

Val Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg
-20                  -15                  -10                   -5

Arg Ala Lys Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys
                 1                    5                   10

Asn Lys Asn Cys Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val
          15                   20                   25

Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr
      30                   35                   40

Cys His Gly Asp Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr
 45                   50                   55                   60

Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile
                65                   70                   75

Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu
             80                   85                   90

Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met
          95                  100                  105

Val Val Glu Gly Cys Gly Cys Arg
   110                  115
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATGGGCAGC TCGAG 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCAGGCGA GCCTGAATTC CTCGAGCCAT CATG 34

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGAGGTTAAA AAACGTCTAG GCCCCCCGAA CCACGGGGAC GTGGTTTTCC TTTGAAAAAC 60

ACGATTGC 68

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 470 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(H) CELL LINE: W138 (genomic DNA)

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: human genomic library
(B) CLONE: lambda 111-1

(viii) POSITION IN GENOME:
(C) UNITS: bp

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..470

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..456

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 124..453

(ix) FEATURE:
(A) NAME/KEY: mRNA
(B) LOCATION: 1..470

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
TGA ACA AGA GAG TGC TCA AGA AGC TGT CCA AGG ACG GCT CCA CAG AGG        48
    Thr Arg Glu Cys Ser Arg Ser Cys Pro Arg Thr Ala Pro Gln Arg
-41 -40                 -35                 -30

CAG GTG AGA GCA GTC ACG AGG AGG ACA CGG ATG GCG CAC GTG GCT GCG        96
Gln Val Arg Ala Val Thr Arg Arg Thr Arg Met Ala His Val Ala Ala
-25                 -20                 -15                 -10

GGG TCG ACT TTA GCC AGG CGG AAA AGG AGC GCC GGG GCT GGC AGC CAC       144
Gly Ser Thr Leu Ala Arg Arg Lys Arg Ser Ala Gly Ala Gly Ser His
                -5                  1                   5

TGT CAA AAG ACC TCC CTG CGG GTA AAC TTC GAG GAC ATC GGC TGG GAC       192
Cys Gln Lys Thr Ser Leu Arg Val Asn Phe Glu Asp Ile Gly Trp Asp
        10                  15                  20

AGC TGG ATC ATT GCA CCC AAG GAG TAT GAA GCC TAC GAG TGT AAG GGC       240
Ser Trp Ile Ile Ala Pro Lys Glu Tyr Glu Ala Tyr Glu Cys Lys Gly
    25                  30                  35

GGC TGC TTC TTC CCC TTG GCT GAC GAT GTG ACG CCG ACG AAA CAC GCT       288
Gly Cys Phe Phe Pro Leu Ala Asp Asp Val Thr Pro Thr Lys His Ala
40                  45                  50                  55

ATC GTG CAG ACC CTG GTG CAT CTC AAG TTC CCC ACA AAG GTG GGC AAG       336
Ile Val Gln Thr Leu Val His Leu Lys Phe Pro Thr Lys Val Gly Lys
                60                  65                  70

GCC TGC TGT GTG CCC ACC AAA CTG AGC CCC ATC TCC GTC CTC TAC AAG       384
Ala Cys Cys Val Pro Thr Lys Leu Ser Pro Ile Ser Val Leu Tyr Lys
            75                  80                  85

GAT GAC ATG GGG GTG CCC ACC CTC AAG TAC CAT TAC GAG GGC ATG AGC       432
Asp Asp Met Gly Val Pro Thr Leu Lys Tyr His Tyr Glu Gly Met Ser
        90                  95                  100

GTG GCA GAG TGT GGG TGC AGG TAGTATCTGC CTGCGGG                        470
Val Ala Glu Cys Gly Cys Arg
    105                 110
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 150 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
    Thr Arg Glu Cys Ser Arg Ser Cys Pro Arg Thr Ala Pro Gln Arg
-41 -40                 -35                 -30

Gln Val Arg Ala Val Thr Arg Arg Thr Arg Met Ala His Val Ala Ala
-25                 -20                 -15                 -10

Gly Ser Thr Leu Ala Arg Arg Lys Arg Ser Ala Gly Ala Gly Ser His
                -5                  1                   5

Cys Gln Lys Thr Ser Leu Arg Val Asn Phe Glu Asp Ile Gly Trp Asp
        10                  15                  20
```

```
Ser Trp Ile Ile Ala Pro Lys Glu Tyr Glu Ala Tyr Glu Cys Lys Gly
 25                  30                  35

Gly Cys Phe Phe Pro Leu Ala Asp Asp Val Thr Pro Thr Lys His Ala
 40                  45                  50                  55

Ile Val Gln Thr Leu Val His Leu Lys Phe Pro Thr Lys Val Gly Lys
                 60                  65                  70

Ala Cys Cys Val Pro Thr Lys Leu Ser Pro Ile Ser Val Leu Tyr Lys
             75                  80                  85

Asp Asp Met Gly Val Pro Thr Leu Lys Tyr His Tyr Glu Gly Met Ser
         90                  95                 100

Val Ala Glu Cys Gly Cys Arg
 105                 110
```

What is claimed is:

1. A BMP-9 polypeptide comprising the amino acid sequence from amino acid #8 - 110 as set forth in FIG. 3 (SEQ ID NO: 9).

2. A BMP-9 polypeptide comprising the amino acid sequence from amino acid #1 - 110 as set forth in FIG. 3 (SEQ ID NO: 9).

3. A BMP-9 polypeptide of claim 1 wherein said polypeptide is a dimer wherein each subunit comprises at least the amino acid sequence from amino acid #8 - 110 of FIG. 3 (SEQ ID NO: 9).

4. A BMP-9 polypeptide of claim 2 wherein said polypeptide is a dimer wherein each subunit comprises at least the amino acid sequence from amino acid #1-110 of FIG. 3. (SEQ ID NO: 9).

5. A purified BMP-9 protein produced by the steps of

(a) culturing a cell transformed with a cDNA comprising the nucleotide sequence from nucleotide #124 to #453 as shown in FIG. 3 (SEQ ID NO: 8); and

(b) recovering and purifying from said culture medium a protein comprising the amino acid sequence from amino acid #1 to amino acid #110 as shown in FIG. 3 (SEQ ID NO: 9).

6. A purified BMP-9 protein produced by the steps of

(a) culturing a cell transformed with a cDNA comprising the nucleotide sequence from nucleotide #124 to #453 as shown in FIG. 3 (SEQ ID NO: 8); and

(b) recovering form said culture medium a protein comprising an amino acid sequence from amino acid #8 to amino acid #110 as shown in Figure 3 (SEQ ID NO: 9).

7. A BMP-9 protein characterized by the ability to induce the formation of cartilage and/or bone.

8. A DNA sequence encoding a BMP-9 protein.

9. The DNA sequence of claim 8 wherein said DNA comprises

(a) nucleotide 124 to 453 (SEQ ID NO: 8); and

(b) sequences which hybridize thereto under stringent hybridization conditions and exhibit the ability to form cartilage and/or bone.

10. The DNA sequence of claim 8 wherein said DNA comprises

(a) nucleotide 145 to 453 (SEQ ID NO: 8); and

(b) sequences which hybridize thereto under stringent hybridization conditions and exhibit the ability to form cartilage and/or bone.

11. A host cell transformed with a DNA sequence encoding BMP-8.

12. A method for producing a purified BMP-9 protein said method comprising the steps of

(a) culturing a cell transformed with a cDNA comprising the nucleotide sequence encoding a BMP-9 protein; and

(b) recovering and purifying said BMP-9 protein from the culture medium.

13. A pharmaceutical composition comprising an effective amount of a BMP-9 protein in admixture with a pharmaceutically acceptable vehicle.

14. A composition of claim 13 further comprising a matrix for supporting said composition and providing a surface for bone and/or cartilage growth.

15. The composition of claim 14 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.

16. A method for inducing bone and/or cartilage formation in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 13.

17. A pharmaceutical composition for wound healing and tissue repair said composition comprising an effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle.

18. A method for treating wounds and/or tissue repair in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 17.

19. A purified mammalian BMP-9 protein produced by the steps of

(a) culturing a cell transformed with (i) a DNA comprising the nucleotide sequence from nucleotide #610 to #1893 of SEQ ID NO:1 and (ii) sequences which hybridize thereto under stringent hybridization conditions and induces the formation of cartilage or bone; and

(b) recovering and purifying from said culture medium a protein comprising amino acid #1 to #110 of SEQ ID NO:9.

20. A pharmaceutical composition for hepatocyte growth said composition comprising an effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle.

21. A method for inducing hepatocyte growth in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 20.

22. A pharmaceutical composition for cartilage repair said composition comprising an effective amount of a BMP-9 protein in a pharmaceutically acceptable vehicle.

Figure 1/1

```
                10        20        30        40        50        60        70
CATTAATAAA TATTAAGTAT TGGAATTAGT GAAATTGGAG TTCCTTGTGG AAGGAAGTGG GCAAGTGAGC
        80        90       100       110       120       130       140
TTTTTAGTTT GTGTCGGAAG CCTGTAATTA CGGCTCCAGC TCATAGTGGA ATGGCTATAC TTAGATTTAT
       150       160       170       180       190       200       210
GGATAGTTGG GTAGTAGGTG TAAATGTATG TGGTAAAAGG CCTAGGAGAT TTGTTGATCC AATAAATATG
       220       230       240       250       260       270       280
ATTAGGGAAA CAATTATTAG GGTTCATGTT CGTCCTTTTG GTGTGTGGAT TAGCATTATT TGTTTGATAA
       290       300       310       320       330       340       350
TAAGTTTAAC TAGTCAGTGT TGGAAAGAAT GGAGACGGTT GTTGATTAGG CGTTTTGAGG ATGGGAATAG
       360       370       380       390       400       410       420
GATTGAAGGA AATATAATGA TGGCTACAAC GATTGGGAAT CCTATTATTG TTGGGGTAAT GAATGAGGCA
       430       440       450       460       470       480       490
AATAGATTTT CGTTCATTTT AATTCTCAAG GGGTTTTTAC TTTTATGTTT GTTAGTGATA TTGGTGAGTA
       500       510       520       530       540       550       560
GGCCAAGGGT TAATAGTGTA ATTGAATTAT AGTGAAATCA TATTACTAGA CCTGATGTTA GAAGGAGGGC
       570       580       590       600       609       618
                                          >__ ___ ___ ___ ___
TGAAAAGGCT CCTTCCCTCC CAGGACAAAA CCGGAGCAGG GCCACCCGG ATG TCC CCT GGG
                                                  M   S   P   G
```

Figure 1/2

```
627                636                645                654                663                672
GCC TTC CGG GTG GCC CTG CTC CCG CTG TTC CTG CTG GTC TGT GTC ACA CAG CAG
A   F   R   V   A   L   L   P   L   F   L   L   V   C   V   T   Q   Q

681                690                699                708                717                726
AAG CCG CTG CAG AAC TGG GAA CAA GCA TCC CCT GGG GAA AAT GCC CAC AGC TCC
K   P   L   Q   N   W   E   Q   A   S   P   G   E   N   A   H   S   S

735                744                753                762                771                780
CTG GGA TTG TCT GGA GCT GGA GAG GAG GGT GTC TTT GAC CTG CAG ATG TTC CTG
L   G   L   S   G   A   G   E   E   G   V   F   D   L   Q   M   F   L

789                798                807                816                825                834
GAG AAC ATG AAG GTG GAT TTC CTA CGC AGC CTT AAC CTC AGC GGC ATT CCC TCC
E   N   M   K   V   D   F   L   R   S   L   N   L   S   G   I   P   S
```

Figure 1/3

```
                843                 852                 861                 870                 879                 888
                ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___
                CAG GAC AAA ACC AGA GCG GAG CCA CCC CAG TAC ATG ATC GAC TTG TAC AAC AGA
                Q   D   K   T   R   A   E   P   P   Q   Y   M   I   D   L   Y   N   R

                897                 906                 915                 924                 933                 942
                ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___
                TAC ACA ACG GAC AAA TCG TCT ACG CCT GCC TCC AAC ATC GTG CGG AGC TTC AGC
                Y   T   T   D   K   S   S   T   P   A   S   N   I   V   R   S   F   S

                951                 960                 969                 978                 987                 996
                ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___
                GTG GAA GAT GCT ATA TCG ACA GCT GCC ACG GAG GAC TTC CCC TTT CAG AAG CAC
                V   E   D   A   I   S   T   A   A   T   E   D   F   P   P   Q   K   H

                1005                1014                1023                1032                1041                1050
                ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___
                ATC CTG ATC TTC AAC ATC TCC ATC CCG AGG CAC GAG CAG ATC ACC AGG GCT GAG
                I   L   I   F   N   I   S   I   P   R   H   E   Q   I   T   R   A   E
```

Figure 1/4

```
1059        1068        1077        1086        1095        1104
 |           |           |           |           |           |
CTC CGA CTC TAT GTC TCC TGC CAA AAT GAT GTG GAC TCC ACT CAT GGG CTG GAA
L   R   L   Y   V   S   C   Q   N   D   V   D   S   T   H   G   L   E

1113        1122        1131        1140        1149        1158
 |           |           |           |           |           |
GGA AGC ATG GTC GTT TAT GAT GTT CTG GAG GAC AGT GAG ACT TGG GAC CAG GCC
G   S   M   V   V   Y   D   V   L   E   D   S   E   T   W   D   Q   A

1167        1176        1185        1194        1203        1212
 |           |           |           |           |           |
ACG GGG ACC AAG ACC TTC TTG GTA TCC CAG GAC ATT CGG GAC GAA GGA TGG GAG
T   G   T   K   T   F   L   V   S   Q   D   I   R   D   E   G   W   E

1221        1230        1239        1248        1257        1266
 |           |           |           |           |           |
ACT TTA GAA GTA TCG AGT GCC GTG AAG CGG TGG GTC AGG GCA GAC TCC ACA ACA
T   L   E   V   S   S   A   V   K   R   W   V   R   A   D   S   T   T
```

Figure 1/5

```
1275        1284        1293        1302        1311        1320
AAC AAA AAT AAG CTC GAG GTG ACA GTG CAG AGC CAC AGG GAG AGC TGT GAC ACA
N   K   N   K   L   E   V   T   V   Q   S   H   R   E   S   C   D   T

1329        1338        1347        1356        1365        1374
CTG GAC ATC AGT GTC CCT CCA GGT TCC AAA AAC CTG CCC TTC TTT GTT GTC TTC
L   D   I   S   V   P   P   G   S   K   N   L   P   F   F   V   V   F

1383        1392        1401        1410        1419        1428
TCC AAT GAC CGC AGC AAT GGG ACC AAG GAG ACC AGA CTG GAG CTG AAG GAG ATG
S   N   D   R   S   N   G   T   K   E   T   R   L   E   L   K   E   M

1437        1446        1455        1464        1473        1482
ATC GGC CAT GAG CAG GAG ACC ATG CTT GTG AAG ACA GCC AAA AAT GCT TAC CAG
I   G   H   E   Q   E   T   M   L   V   K   T   A   K   N   A   Y   Q
```

Figure 1/6

```
                1491        1500        1509        1518        1527        1536
                 |           |           |           |           |           |
GTG GCA GGT GAG AGC CAA GAG GAG GAG GGT CTA GAT GGA TAC ACA GCT GTG GGA
V   A   G   E   S   Q   E   E   E   G   L   D   G   Y   T   A   V   G

                1545        1554        1563        1572        1581        1590
                 |           |           |           |           |           |
CCA CTT TTA GCT AGA AGG AAG AGG AGC ACC GGA GCC AGC AGC CAC TGC CAG AAG
P   L   L   A   R   R   K   R   S   T   G   A   S   S   H   C   Q   K
                                (319)                       (326)

                1599        1608        1617        1626        1635        1644
                 |           |           |           |           |           |
ACT TCT CTC AGG GTG AAC TTT GAG GAC ATC GGC TGG GAC AGC TGG ATC ATT GCA
T   S   L   R   V   N   F   E   D   I   G   W   D   S   W   I   I   A

                1653        1662        1671        1680        1689        1698
                 |           |           |           |           |           |
CCC AAG GAA TAT GAC GCC TAT GAG TGT AAA GGG GGT TGC TTC TTC CCA TTG GCT
P   K   E   Y   D   A   Y   E   C   K   G   G   C   F   F   P   L   A
```

Figure 1/7

```
   1707        1716        1725        1734        1743        1752
GAT GAC GTG ACA CCC ACC AAA CAT GCC ATC GTG CAG ACC CTG GTG CAT CTC GAG
D   D   V   T   P   T   K   H   A   I   V   Q   T   L   V   H   L   E

   1761        1770        1779        1788        1797        1806
TTC CCC ACA AAG GTG GGC AAA GCC TGC TGC GTT CCC ACC AAA CTG AGT CCC ATC
F   P   T   K   V   G   K   A   C   C   V   P   T   K   L   S   P   I

   1815        1824        1833        1842        1851        1860
TCC ATC CTC TAC AAG GAT GAC ATG GGG GTG CCA ACC CTC AAG TAC CAC TAT GAG
S   I   L   Y   K   D   D   M   G   V   P   T   L   K   Y   H   Y   E

   1869        1878        1887                1903        1913        1923
GGG ATG AGT GTG GCT GAG TGT GGG TGT AGG TAGTCCCTGC AGCCACCCAG GGTGGGGATA
G   M   S   V   A   E   C   G   C   R
                                    (428)
```

Figure 1/8

```
1933  1943  1953  1963  1973  1983  1993
CAGGACATGG AAGAGGTTCT GGTACGGTCC TGCATCCTCC TGCGCATGGT ATGCCTAAGT TGATCAGAAA
2003  2013  2023  2033  2043  2053  2063
CCATCCTTGA GAAGAAAAGG AGTTAGTTGC CCTTCTTGTG TCTGGTGGGT CCCTCTGCTG AAGTGACAAT
2073  2083  2093  2103  2113  2123  2133
GACTGGGGTA TGCGGGCCTG TGGGCAGAGC AGGAGACCCT GGAAGGGTTA GTGGGTAGAA AGATGTCAAA
2143  2153  2163  2173  2183  2193  2203
AAGGAAGCTG TGGGTAGATG ACCTGCACTC CAGTGATTAG AAGTCCAGCC TTACCTGTGA GAGAGCTCCT
2213  2223  2233  2243  2253  2263  2273
GGCATCTAAG AGAACTCTGC TTCCTCATCA TCCCCACCGA CTTGTTCTTC CTTGGGAGTG TGTCCTCAGG
2283  2293  2303  2313  2323  2333  2343
GAGAACAGCA TTGCTGTTCC TGTGCCTCAA GCTCCCAGCT GACTCTCCTG TGGCTCATAG GACTGAATGG
2353  2363  2373  2383  2393  2403  2413
GGTGAGGAAG AGCCTGATGC CCTCTGGCAA TCAGAGCCCG AAGGACTTCA AAACATCTGG ACAACTCTCA
2423  2433  2443
TTGACTGATG CTCCAACATA ATTTTTAAAA AGAG
```

Figure 2/1

```
        10          20          30          40          50          60          70
CTCTAGAGGG CAGAGGAGGA GGGAGGGAGG GAAGGAGCGC GGAGCCCGGC CCGGAAGCTA GGTGAGTGTG

        80          90         100         110         120         130         140
GCATCCGAGC TGAGGGACGC GAGCCTGAGA CGCCGCTGCT GCTCCGGCTG AGTATCTAGC TTGTCTCCCC

       150         160         170         180         190         200         210
GATGGGATTC CCGTCCAAGC TATCTCGAGC CTGCAGCGCC ACAGTCCCCG GCCCTCGCCC AGGTTCACTG

       220         230         240         250         260         270         280
CAACCGTTCA GAGGTCCCCA GGAGCTGCTG CTGGCGAGCC CGCTACTGCA GGGACCTATG GAGCCATTCC

       290         300         310         320         330         340         350
GTAGTGCCAT CCCGAGCAAC GCACTGCTGC AGCTTCCCTG AGCCTTTCCA GCAAGTTTGT TCAAGATTGG

       360         370         380         390         400    (1)
CTGTCAAGAA TCATGGACTG TTATTATATG CCTTGTTTTC TGTCAAGACA CC ATG ATT CCT
                                                           MET Ile Pro

   417                 432                 447                 462
GGT AAC CGA ATG CTG ATG GTC GTT TTA TTA TGC CAA GTC CTG CTA GGA GGC GCG
Gly Asn Arg MET Leu MET Val Val Leu Leu Cys Gln Val Leu Leu Gly Gly Ala
```

Figure 2/2

```
            477                 492                 507
AGC CAT GCT AGT TTG ATA CCT GAG ACG GGG AAG AAA AAA GTC GCC GAG ATT CAG
Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys Lys Lys Val Ala Glu Ile Gln

522                 537                 552                 567
GGC CAC GCG GGA GGA CGC CGC TCA GGG CAG AGC CAT GAG CTC CTG CGG GAC TTC
Gly His Ala Gly Gly Arg Arg Ser Gly Gln Ser His Glu Leu Leu Arg Asp Phe

        582                 597                 612                 627
GAG GCG ACA CTT CTG CAG ATG TTT GGG CTG CGC CGC CGC CCG CAG CCT AGC AAG
Glu Ala Thr Leu Leu Gln MET Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys

                642                 657                 672
AGT GCC GTC ATT CCG GAC TAC ATG CGG GAT CTT TAC CGG CTT CAG TCT GGG GAG
Ser Ala Val Ile Pro Asp Tyr MET Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu

    687                 702                 717                 732
GAG GAG GAA GAG CAG ATC CAC AGC ACT GGT CTT GAG TAT CCT GAG CGC CCG GCC
Glu Glu Glu Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala
```

Figure 2/3

```
                747                 762                 777
AGC CGG GCC AAC ACC GTG AGG AGC TTC CAC CAC GAA GAA CAT CTG GAG AAC ATC
Ser Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn Ile

            792                 807                 822                 837
CCA GGG ACC AGT GAA AAC TCT GCT TTT CGT TTC CTC TTT AAC CTC AGC AGC ATC
Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu Ser Ser Ile

        852                 867                 882                 897
CCT GAG AAC GAG GTG ATC TCC TCT GCA GAG CTT CGG CTC TTC CGG GAG CAG GTG
Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg Leu Phe Arg Glu Gln Val

            912                 927                 942
GAC CAG GGC CCT GAT TGG GAA AGG GGC TTC CAC CGT ATA AAC ATT TAT GAG GTT
Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile Tyr Glu Val

    957                 972                 987                1002
ATG AAG CCC CCA GCA GAA GTG GTG CCT GGG CAC CTC ATC ACA CGA CTA CTG GAC
MET Lys Pro Pro Ala Glu Val Val Pro Gly His Leu Ile Thr Arg Leu Leu Asp
```

Figure 2/4

```
          1017                1032                1047
ACG AGA CTG GTC CAC CAC AAT GTG ACA CGG TGG GAA ACT TTT GAT GTG AGC CCT
Thr Arg Leu Val His His Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro

1062                1077                1092                1107
GCG GTC CTT CGC TGG ACC CGG GAG AAG CAG CCA AAC TAT GGG CTA GCC ATT GAG
Ala Val Leu Arg Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu

      1122                1137                1152                1167
GTG ACT CAC CTC CAT CAG ACT CGG ACC CAC CAG GGC CAG CAT GTC AGG ATT AGC
Val Thr His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser

               1182                1197                1212
CGA TCG TTA CCT CAA GGG AGT GGG AAT TGG GCC CAG CTC CGG CCC CTC CTG GTC
Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu Val

  1227                1242                1257                1272
ACC TTT GGC CAT GAT GGC CGG GGC CAT GCC TTG ACC CGA CGC CGG AGG GCC AAG
Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg Arg Ala Lys

          1287                1302                1317
CGT AGC CCT AAG CAT CAC TCA CAG CGG GCC AGG AAG AAG AAT AAG AAC TGC CGG
Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys Arg
```

Figure 2/5

```
1332(311)          1347                1362                1377
CGC CAC TCG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC TGG ATT GTG
Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val

      1392                1407                1422                1437
GCC CCA CCA GGC TAC CAG GCC TTC TAC TGC CAT GGG GAC TGC CCC TTT CCA CTG
Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp Cys Pro Phe Pro Leu

               1452                1467                1482
GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT GTG CAG ACC CTG GTC AAT TCT
Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln Thr Leu Val Asn Ser

   1497                1512                1527                1542
GTC AAT TCC AGT ATC CCC AAA GCC TGT TGT GTG CCC ACT GAA CTG AGT GCC ATC
Val Asn Ser Ser Ile Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile

           1557                1572                1587
TCC ATG CTG TAC CTG GAT GAG TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG
Ser MET Leu Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu
```

Figure 2/6

```
1602          1617          (408)          1636          1646          1656
ATG GTA GTA GAG GGA TGT GGG TGC CGC TGAGATCAGG CAGTCCTTGA GGATAGACAG
MET Val Val Glu Gly Cys Gly Cys Arg

  1666          1676          1686          1696          1706          1716          1726
ATATACACAC CACACACACA CACCACATAC ACCACACACA CACGTTCCCA TCCACTCACC CACACTAC

  1736          1746          1756          1766          1776          1786          1796
ACAGACTGCT TCCTTATAGC TGGACTTTTA TTTAAAAAAA AAAAAAAAAA AATGGAAAAA ATCCCTAAAC

  1806          1816          1826          1836          1846          1856          1866
ATTCACCTTG ACCTTATTTA TGACTTTACG TGCAAATGTT TTGACCATAT TGATCATATA TTTTGACAAA

  1876          1886          1896          1906          1916          1926          1936
ATATATTTAT AACTACGTAT TAAAAGAAAA AAATAAAATG AGTCATTATT TTAAAAAAAA AAAAAAACT

  1946
CTAGAGTCGA CGGAATTC
```

Figure 3

```
TGA ACA AGA GAG TGC TCA AGA AGC TGT CCA AGG ACG GCT CCA CAG AGG      48
 *  Thr Arg Glu Cys Ser Arg Ser Cys Pro Arg Thr Ala Pro Gln Arg
-41 -40                 -35                 -30

CAG GTG AGA GCA GTC ACG AGG AGG ACA CGG ATG GCG CAC GTG GCT GCG      96
Gln Val Arg Ala Val Thr Arg Arg Thr Arg Met Ala His Val Ala Ala
-25                 -20                 -15                 -10

GGG TCG ACT TTA GCC AGG CGG AAA AGG AGC GCC GGG GCT GGC AGC CAC     144
Gly Ser Thr Leu Ala Arg Arg Lys Arg Ser Ala Gly Ala Gly Ser His
                -5                   1                   5

TGT CAA AAG ACC TCC CTG CGG GTA AAC TTC GAG GAC ATC GGC TGG GAC     192
Cys Gln Lys Thr Ser Leu Arg Val Asn Phe Glu Asp Ile Gly Trp Asp
         10                  15                  20

AGC TGG ATC ATT GCA CCC AAG GAG TAT GAA GCC TAC GAG TGT AAG GGC     240
Ser Trp Ile Ile Ala Pro Lys Glu Tyr Glu Ala Tyr Glu Cys Lys Gly
     25                  30                  35

GGC TGC TTC TTC CCC TTG GCT GAC GAT GTG ACG CCG ACG AAA CAC GCT     286
Gly Cys Phe Phe Pro Leu Ala Asp Asp Val Thr Pro Thr Lys His Ala
 40                  45                  50                  55

ATC GTG CAG ACC CTG GTG CAT CTC AAG TTC CCC ACA AAG GTG GGC AAG     336
Ile Val Gln Thr Leu Val His Leu Lys Phe Pro Thr Lys Val Gly Lys
                60                  65                  70

GCC TGC TGT GTG CCC ACC AAA CTG AGC CCC ATC TCC GTC CTC TAC AAG     384
Ala Cys Cys Val Pro Thr Lys Leu Ser Pro Ile Ser Val Leu Tyr Lys
            75                  80                  85

GAT GAC ATG GGG GTG CCC ACC CTC AAG TAC CAT TAC GAG GGC ATG AGC     432
Asp Asp Met Gly Val Pro Thr Leu Lys Tyr His Tyr Glu Gly Met Ser
        90                  95                 100

GTG GCA GAG TGT GGG TGC AGG TAGTATCTGC CTGCGGG                      470
Val Ala Glu Cys Gly Cys Arg
    105                 110
```

Figure 4

SULFATE INCORPORATION

Bovine Explants

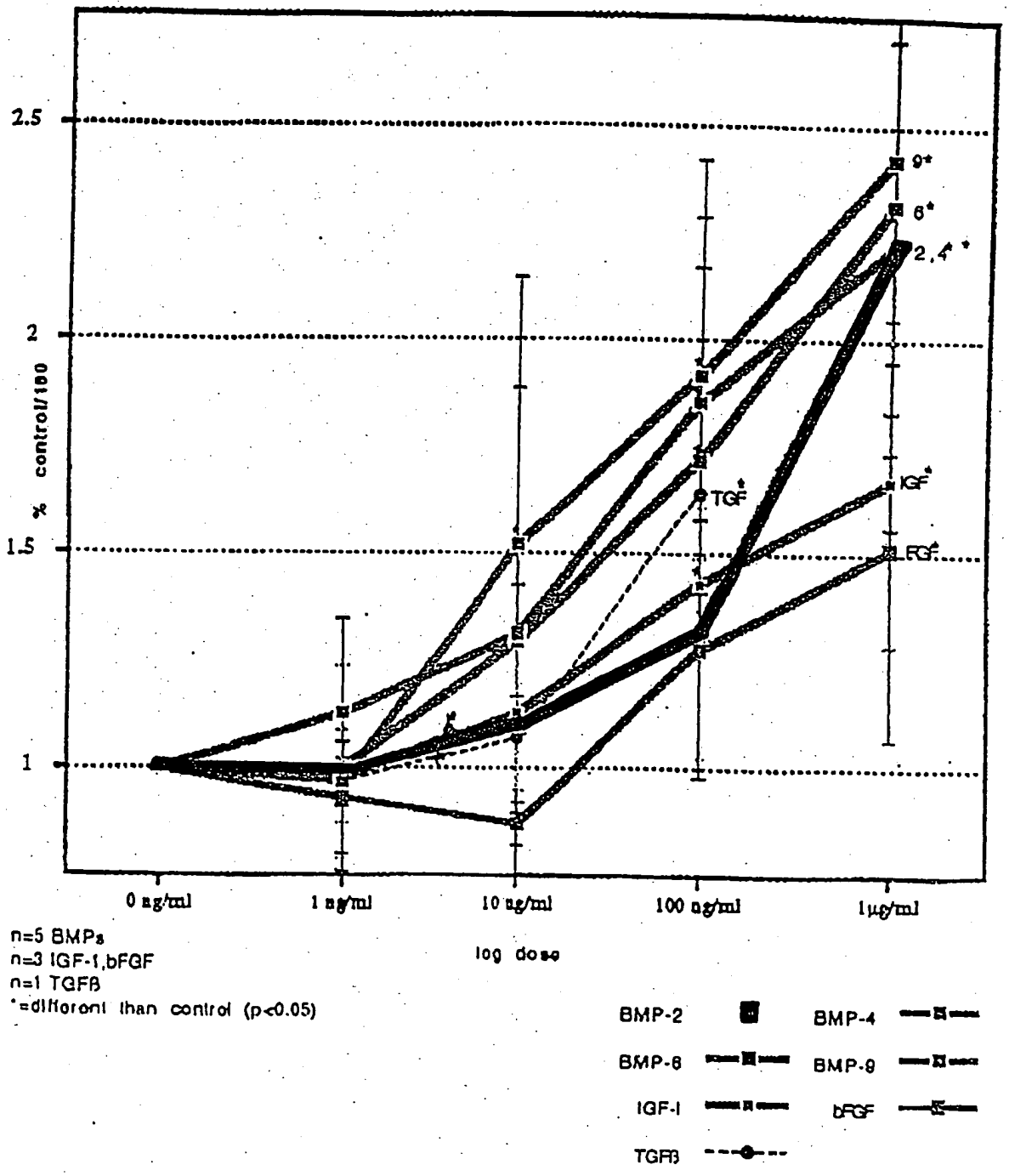


Figure 5

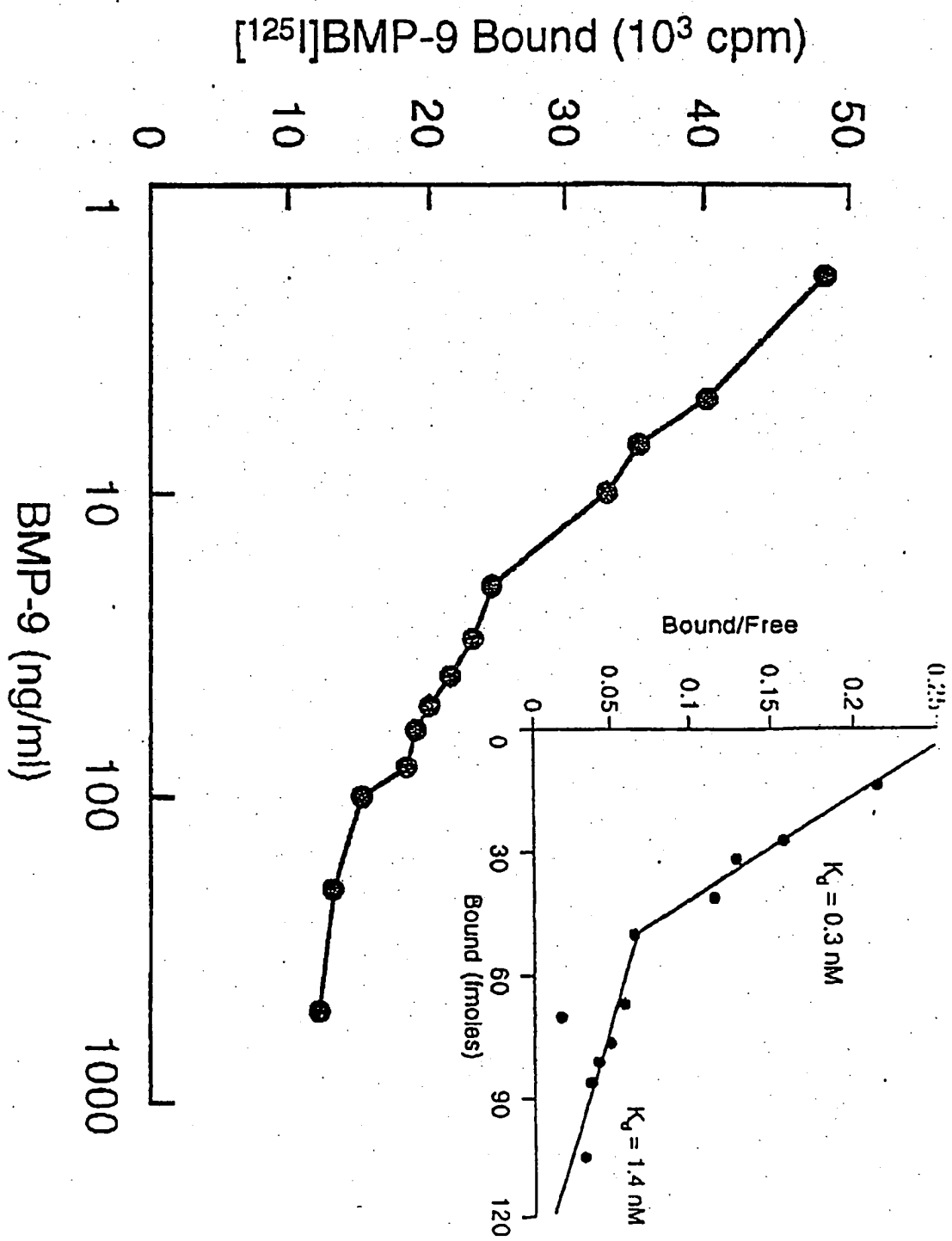

[125I]BMP-9 Bound (10^3 cpm)
0
10
20
30
40
50
1
10
100
1000
BMP-9 (ng/ml)
Bound/Free
0
0.05
0.1
0.15
0.2
0
30
60
90
120
Bound (fmoles)
Kd = 0.3 nM
Kd = 1.4 nM

Figure 6/1

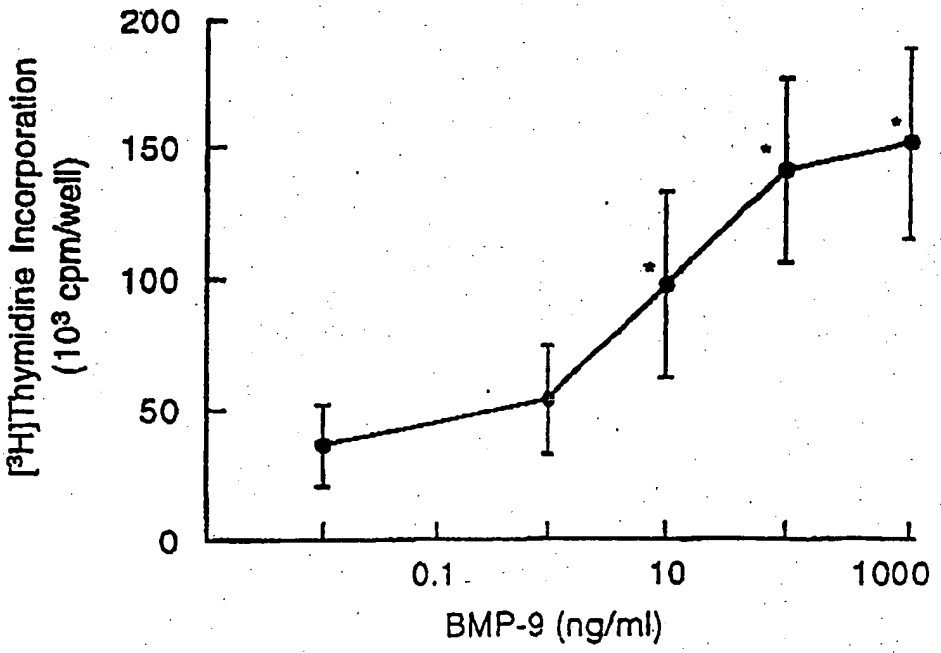

200
150
100
50
0
[³H]Thymidine Incorporation
(10³ cpm/well)
0.1
10
1000
BMP-9 (ng/ml)
*
*
*

Figure 6/2

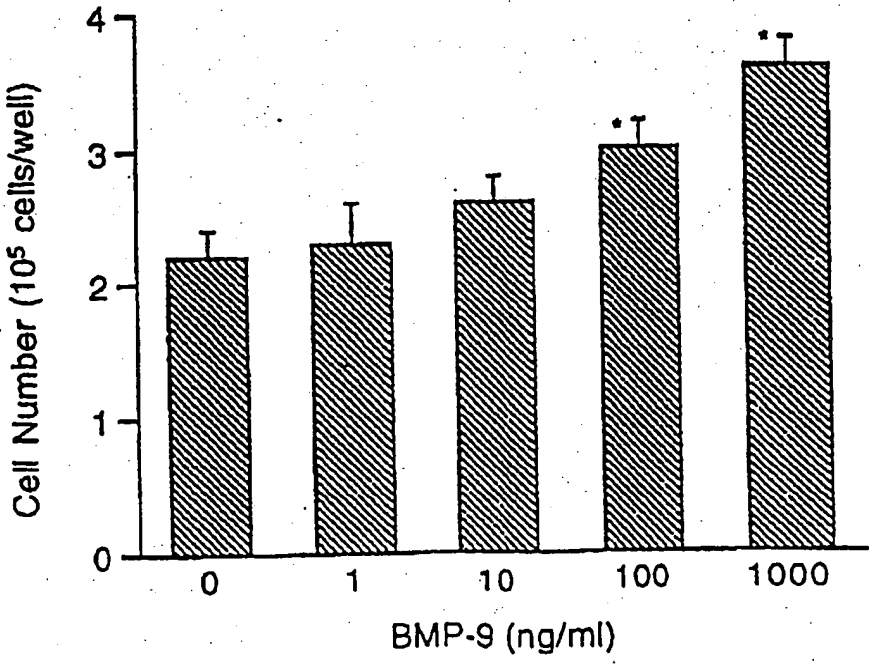
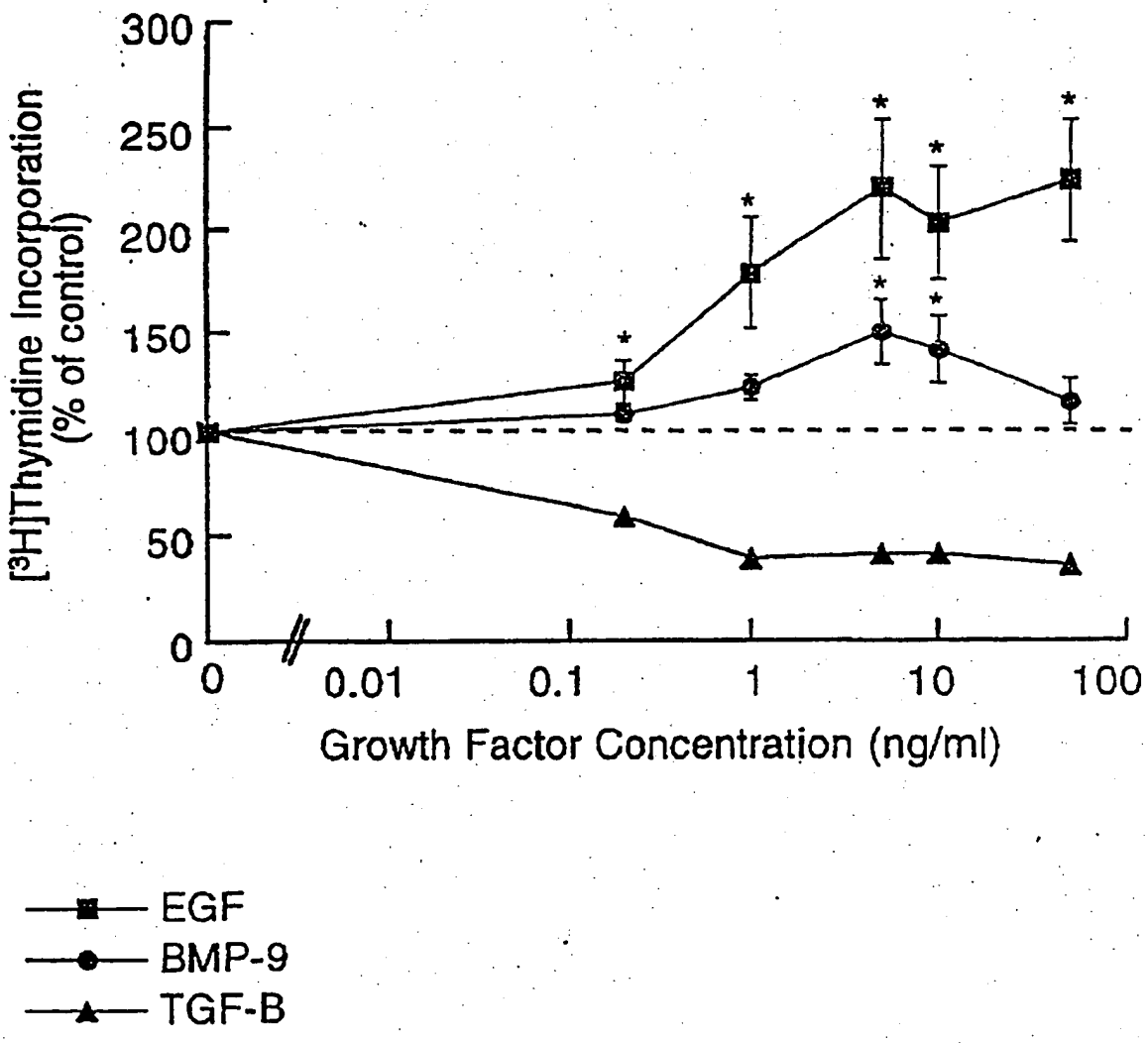


Figure 7


[³H]Thymidine Incorporation (% of control)
300
250
200
150
100
50
0
0
0.01
0.1
1
10
100
Growth Factor Concentration (ng/ml)
EGF
BMP-9
TGF-B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/07084

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/51 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 00432 (GENETICS INST) 7 January 1993 see the whole document ---	7,8, 11-18,22
Y		20,21
Y	WO,A,94 06449 (CREATIVE BIOMOLECULES INC) 31 March 1994 see the whole document ---	20,21
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 1995	16.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 95/07084

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,P, X	MOLECULAR BIOLOGY OF THE CELL, vol. 5, October 1994 page 384a SONG, J. ET AL. 'Bone morphogenetic protein-9 (BMP-9) binds to HEPG2 cells and stimulates proliferation' see abstract & 34th Ann.Meet. of the American Soc. for Cell Biol.; december 10-14, 1994; San Francisco, California	20,21

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07084

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 16,18,21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the aaleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 11 refering to a DNA sequence encoding BMP-8, has been interpreted as being meant to refer to BMP-9!

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/07084

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9300432	07-01-93	AU-B-	652472	25-08-94
		AU-A-	2269992	25-01-93
		EP-A-	0592562	20-04-94
		JP-T-	6508990	13-10-94
WO-A-9406449	31-03-94	AU-B-	4795193	03-03-94
		AU-B-	4797193	03-03-94
		AU-B-	4995593	03-03-94
		AU-B-	5129293	12-04-94
		AU-B-	5129393	12-04-94
		AU-B-	5162393	12-04-94
		AU-B-	5290893	12-04-94
		AU-B-	5590094	24-05-94
		CA-A-	2141554	17-02-94
		CA-A-	2141555	17-02-94
		CA-A-	2141556	17-02-94
		CA-A-	2147598	11-05-94
		EP-A-	0652953	17-05-95
		EP-A-	0653942	24-05-95
		EP-A-	0661933	12-07-95
		EP-A-	0665739	09-08-95
		EP-A-	0661987	12-07-95
		EP-A-	0672064	20-09-95
		WO-A-	9403600	17-02-94
		WO-A-	9403075	17-02-94
		WO-A-	9403200	17-02-94
		WO-A-	9406447	31-03-94
		WO-A-	9406399	31-03-94
		WO-A-	9406420	31-03-94
		WO-A-	9410203	11-05-94